**Enhancing the Nitrite Reductase Activity of Modified
Hemoglobin: Bis-Tetramers and their PEGylated Derivatives**

by

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Abstract

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The need for an alternative to red cells in transfusions has led to the creation of hemoglobin-based oxygen carriers (HBOCs). However, evaluations of all products tested in clinical trials have noted cardiovascular complications, raising questions about their safety that led to the abandonment of all those products. It has been considered that the adverse side effects come from the scavenging of the vasodilator – nitric oxide (NO) by the deoxyheme sites of the hemoglobin derivatives. Another observation is that HBOCs with lower oxygen affinity than red cells release oxygen prematurely in arterioles, triggering an unwanted homeostatic response. Since the need for such a product remains critical, it is important to understand the reactivity patterns that contribute to the observed complications.

Various alterations of the protein have been attempted in order to reduce HBOC-induced vasoconstriction. Recent reports suggest that a safe and effective product should be pure, homogenous and have a high molecular weight along with appropriate oxygenation properties. While these properties are clearly important, vasodilatory features of hemoglobin through its nitrite reductase activity may also act as an *in situ* source of NO. It follows that HBOCs with an enhanced ability to produce NO from endogenous nitrite may serve to counteract vasoactivity associated with NO-scavenging by hemoglobin.

Here we characterize the effects of different protein modifications on the nitrite reductase activity of hemoglobin. We produced a variety of HBOCs that include cross-linked tetramers, polyethylene glycol (PEG) conjugates and bis-tetramers of hemoglobin. We report that the rate of NO production strongly depends on the conformational state of the protein, with R-state stabilized proteins (PEG-Hbs), exhibiting the fastest rates. In particular, we found that PEGylated bis-tetramers of hemoglobin (BT-PEG) exhibit increased nitrite reductase activity while retaining cooperativity and stability. Animal studies of BT-PEG demonstrated that this material is benign: it did not cause significant increases in systemic blood pressure in mice, the major side effect associated with existing HBOCs. BT-PEG exhibits an enhanced nitrite reductase activity together with sample purity and homogeneity, molecular size and shape, and appropriate oxygenation properties, characteristics of a clinically useful HBOC.

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For M.N.V

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Abbreviations

Hb	Hemoglobin
RBC	Red blood cell
PEG	Polyethylene glycol
PEGylation	Conjugation of PEG chains to proteins
HBOC	Hb based oxygen carrier
SFH-P	Stroma-free human Hb product (PolyHeme, Northfield Laboratories)
HBOC-201	Glutaraldehyde bovine Hb (Hemopure, Biopure Corporation)
MP4OX	Hexa-PEG conjugated Hb (Hemospan, Sangart, Inc)
ATP	Adenosine triphosphate
P ₅₀	Oxygen pressure at which Hb is half saturated
<i>n</i> ₅₀	Hill's coefficient of cooperativity at half saturation
NO	Nitric oxide
BPG	2,3-Bisphosphoglycerate
COHb	Carbonmonoxyhemoglobin
DeoxyHb	Deoxygenated hemoglobin
oxyHb	Oxygenated hemoglobin
metHb	Methemoglobin
NOHb	Nitrosylhemoglobin
Mal-PEG5K	Methoxypolyethylene-glycol-(5000)-maleimide
DTNB	5,5'-dithiobis(2-nitrobenzoate)
CD	Circular dichroism
NEM	<i>N</i> -ethyl maleimide
DBS	Di-bromosalicylate
DBSF	3,5-dibromosalicyl fumarate

DMSO	Dimethylsulfoxide
Rp-HPLC	Reverse-phase-high performance liquid chromatography
UV-Vis	Ultraviolet-visible
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Bis-Tris	Bis-(2-hydroxy-ethyl)- amino-tris(hydroxymethyl)-methane
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
$\alpha\alpha$ -Hb	$\alpha\alpha$ -cross-linked hemoglobin
$\alpha\alpha$ -Hb-2PEG	$\alpha\alpha$ -diasprin cross-linked, β -Cys93 Mal-PEG5K conjugated hemoglobin
Hb-2PEG	β -Cys93 Mal-PEG5K conjugated hemoglobin
Hb-6PEG	Thiol-mediated, Mal-PEG5K conjugated hemoglobin
Hb-6NEM	β -Cys93 NEM modified hemoglobin
BT-Hb	Bis-tetramers of hemoglobin
BT-PEG	Bis-tetramers of hemoglobin PEGylated at β -Cys93 with Mal-PEG5K
$k_{\text{obs(NiR)}}$	Bimolecular nitrite-reductase rate constant
NAC	<i>N</i> -acetyl cysteine
WT	Wild type
Db/db	Diabetic
SBP	Systolic blood pressure
HR	Heart rate
SD	Standard deviation
eNOS	Endothelial nitric oxide synthase
mTet	Murine (mouse) tetrameric hemoglobin

Statement of Authorship and Publication Status

Chapter 1

Title: Reviving Artificial Blood: Meeting the Challenge of Dealing with NO Scavenging by Hemoglobin

Authors: Francine E. Lui and Ronald Kluger

Note: This review included in this thesis is an overview of the work detailing the mechanisms of toxicity in HBOC-induced vasoconstriction. The central focus of the review is on hemoglobin's nitrite reductase activity and its role in hypoxic vasodilation. The manuscript was written and edited by Francine E. Lui and Ronald Kluger.

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Authors: Francine E. Lui, Pengcheng Dong and Ronald Kluger

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Chapter 1 : HBOCs – Safety and Efficacy

1.1 Blood, Transfusions, Hemoglobin, and Oxygen Delivery

In blood, red cells (erythrocytes) are responsible for oxygen transport and their rapid depletion can lead to hypoxic organ damage and even to death (1). Transfusions of red blood cells (RBCs) restore circulatory oxygenation capacity, which is reduced following blood loss (2). RBC units derived from blood donors, however, cannot be sterilized and thus have the potential to transmit pathogens (3). They are also subject to rapid aging and present critical requirements for storage (3). In addition, the increasing demand for RBC units cannot be fully supplied. In 2001, 18.9 % of hospitals reported a shortage for non-surgical purposes and 12.7 % reported cancellation of surgeries due to a shortage of blood (4). These problems are expected to increase in the near future (5), supporting the need for blood substitutes. Stabilized hemoglobins, have been produced by chemical modifications and protein engineering as alternatives to red cells in transfusions. These materials are called hemoglobin-based oxygen carriers (HBOCs).

Figure 1.1: Human hemoglobin A is made up of two α -subunits (red) and two β -subunits (blue). Ferrous heme (outlined in green) binds and release oxygen, facilitating respiration in animals.

Human hemoglobin is made up of four protein subunits ($\alpha_2\beta_2$), each with an iron-porphyrin heme center that binds oxygen reversibly (Figure 1.1). Hemoglobin's oxygen binding cooperativity is described by the T- to R-state quaternary conformational shift (Figure 1.2A) (6). There is significant variation between the three-dimensional structure of deoxy-Hb (T-state) and ligated oxy-Hb (R-state), with a 15° shift between the $\alpha\beta$ interfaces of the tetrameric protein (7, 8). The relative stabilities of the T and R states vary with fractional saturation and the sequential binding of ligand progressively induces conformational changes in the subunits (6). This results in allosteric cooperativity, which is traditionally measured as the Hill constant, with $n_{50} = 3$ for native hemoglobin (9). A decrease in n_{50} is indicative of decreased cooperativity.

Figure 1.2: (A) As oxygen binds to the low affinity T-state hemoglobin, conformational changes within the subunit are translated across the quaternary structure, increasing the oxygen affinity in the remaining un-liganded subunits. Hemoglobin's allosteric oxygen binding cooperativity is derived from this $T \rightarrow R$ conformational shift. (B) Oxygen saturation curve of cooperative (dashed line) and non-cooperative (solid line) behaviour.

Cooperative oxygen binding is represented by a sigmoidal curve (Figure 1.2B). An oxygen-binding curve plots partial pressure of oxygen (pO_2) against oxygen saturation (Y) (9). Oxygen affinity (P_{50}) is denoted as the partial pressure at which hemoglobin is half saturated with oxygen. The oxygen affinity is modulated by allosteric effectors that include protons, 2,3-bisphosphoglycerate (BPG), chloride and carbon dioxide (9). The cooperative oxygenation behaviour of hemoglobin is essential to achieve a large extent of loading/unloading capacity of oxygen within a small physiological pressure range (100 torr in arterial blood and 30 torr in venous blood) (9) as depicted by the dashed line in Figure 1.2B. In contrast, derivatives of hemoglobin that lack allosteric cooperativity exhibit a hyperbolic (solid line) oxygen binding curve (Figure 1.2B). Such hemoglobin derivatives release much less of their bound oxygen over the normal physiological range. However, while hemoglobin is the oxygen-carrying protein in red cells, it is unstable outside of the cell and chemical modifications that stabilize the tetrameric state are necessary to prevent dissociation into non-functional $\alpha\beta$ dimers.

1.2 Hemoglobin-Based Oxygen Carriers (HBOCs)

Cross-linking was initially used to stabilize the tetrameric structure ($\alpha_2\beta_2$) of hemoglobin to prevent its spontaneous dissociation. $\alpha\alpha$ -Fumaryl hemoglobin (**DCLHb** “Diaspirin cross-linked hemoglobin”) was initially produced by Klotz and Walder (10). It was tested by the US Army and was advanced to clinical trials by Baxter Healthcare. **DCLHb** is produced by cross-linking the α -subunits within the hemoglobin tetramer by reaction with 3,5-dibromosalicyl fumarate (DBSF) in the presence of a polyanionic effector that blocks the BPG-binding site (Figure 1.4) (11). This gives an $\alpha 99$ - $\alpha 99$ fumaryl lysyl amide cross-linked hemoglobin with a molecular weight of about 64 kDa and $P_{50} = 32$ mmHg and $n_{50} = 2.6$ (11).

Various chemically modified human and bovine hemoglobins have also been produced to serve as potential HBOCs. Initial studies on a variety of potential HBOCs in commercially-oriented clinical trials showed serious side effects, including systemic hypertension, significantly increased risk of myocardial infarction (heart attacks), and increased mortality (12). Specifically, conclusions from a meta-analysis of clinical results

in 2008 led the authors to conclude that infusion of hemoglobin-based oxygen carriers is “associated with a significantly increased risk of death and myocardial infarction” (12). Although these materials may serve as oxygen supplements, assessment of the risks and benefits led the authors to suggest that the trials of these materials be discontinued (12). After this meta-analysis and lack of approval from the United States Food and Drug Administration (FDA) for development of even the most thoroughly tested products, most commercially-directed efforts were discontinued.

1.3 HBOC-Induced Vasoconstriction: Hypotheses

It is thus an important goal to elucidate the origin of the toxicity associated with the clinically tested HBOCs. While the specific cause of the complications remains unclear, one possibility comes from the ability of cell-free hemoglobin to combine tightly with nitric oxide (NO), the endogenous vasodilator. This would be expected to cause constriction of blood vessels. NO is produced from L-arginine by the endothelial enzyme NO synthase (13). It diffuses across the endothelial cells and into the underlying smooth muscle cells, where it stimulates the pathway of cytoplasmic guanylate cyclases to produce vasorelaxation (Figure 1.3) (14).

Figure 1.3: Nitric oxide is produced from NOS in endothelial cells. NO can then diffuse into the blood where it is either taken up by hemoglobin in red blood cells or competes

with superoxide dismutase (SOD) for reaction with superoxide to form peroxynitrite. Alternatively, it can diffuse into vascular smooth muscle cells and bind to guanylate cyclases promoting vasorelaxation. Small molecular weight tetrameric hemoglobin extravasating into smooth muscle cells will deplete NO where it is needed most.

Heme proteins that bind oxygen also bind NO ($k_a \sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (15)) and can thus affect NO homeostasis (16). Hemoglobin within RBCs has limited interaction with NO because the unstirred layer surrounding the erythrocyte membrane allows forms a diffusional barrier between NO and hemoglobin (16). Moreover, the intravascular laminar flow creates an RBC-free zone that consists only of plasma flowing along the endothelium (17). In contrast, tetrameric hemoglobin derivatives are not limited by diffusion through the RBC membrane or the cell-free layer adjacent to vessel walls and are therefore much more effective in scavenging NO (18). This has been proposed to be a potential source of the vasoactivity of hemoglobin derivatives in circulation (16, 19-21).

A separate explanation for the observed vasoconstriction comes from Winslow and co-workers that propose that low affinity HBOCs release oxygen in arterioles, an effect that can induce a vasoconstrictive homeostatic response that limits oxidative stress (22). They conclude that an HBOC with high oxygen affinity (low P_{50}) will instead facilitate delivery of oxygen to sites in the capillaries with low oxygen (23). Originally, it was believed that an HBOC with oxygen affinity close to that of RBCs ($\sim P_{50} = 28 \text{ mmHg}$) will act as a true blood substitute. However the small size of the HBOC means that it has a different mode of oxygen transport from that of RBCs. The decreased hematocrit near vessel walls that result from the RBC-free zone means that there is an increased distance for oxygen to diffuse to tissues from RBCs (97). In contrast, the small size of modified cell-free hemoglobin will allow it to diffuse readily within the lumen, increasing lateral oxygen transport (97). This is the basis of “facilitated diffusion” that is mediated by small, highly diffusible HBOCs that increase lateral transport by acting as carrier proteins (97). Recent studies now indicate that an HBOC with higher oxygen affinity will not only avoid possible adverse homeostatic responses but will also promote movement of oxygen from RBCs to surrounding tissues (18).

The effect of physical stress on the vascular endothelia within specific organs may also serve as a trigger for vasoconstriction (24). Although the shear stress that blood exerts on capillary walls is important for NO production (17), excessive mechanical stress due to turbulent blood flow (instead of laminar flow) could instead, lead to increases in locally-acting effectors, such as angiotensin, endothelin and prostaglandins that signal for contractions of blood vessels (25, 26). It is possible that HBOCs that are subject to laminar flow will exert lower shear stress and may not trigger signals for vasoconstriction.

Despite the noted setbacks in the search for functional HBOCs, the potential for such a material remains a significant and important goal. Methods that deal with reducing NO-scavenging include making larger hemoglobin-centered entities to prevent extravasation and protein-engineered hemoglobins with reduced NO-affinity. In addition, increasing the affinity for oxygen can be achieved by modifying hemoglobin at key residues (e.g. β -Cys93) (9, 27).

Figure 1.4: Chemical reagents used for cross-linking and conjugation of hemoglobin subunits. Reaction of hemoglobin with glutaraldehyde gives polymerized hemoglobin cross-linked at accessible lysine residues producing heterogeneous mixtures of polymerized hemoglobin. In comparison, reaction of hemoglobin with DBSF in the presence of inositol hexaphosphate results in a site-specific cross-link within the $\alpha\alpha'$ -subunits. Surface decoration with inert polymers can also be achieved with maleimide activated polyethylene glycol (Maleimide-PEG).

Increasing molecular weight. The term “extravasation” refers to the leakage of species from the circulatory system into the interstitial space between smooth muscle cells (Figure 1.3). If the low molecular weight cross-linked hemoglobins undergo extravasation, even to a small extent, they could scavenge critical amounts of NO and thus prevent relaxation of the blood vessel, constricting the flow of blood (28). An appropriate macromolecule that is larger than a tetramer might be able to attenuate interactions with the endothelium. Encapsulating the protein (29-31) or increasing its effective size by conjugation or polymerization might provide a means of attenuating the scavenging of NO from the endothelial regions (32-34).

Polyheme, Northfield Laboratories’ polymerized human hemoglobin and **Hemopure**, Biopure Cooperation’s polymerized bovine hemoglobin are examples of glutaraldehyde-treated hemoglobins that have undergone both animal studies and extensive clinical testing (35). **Polyheme** is produced by cross-linking human hemoglobin with glutaraldehyde (Figure 1.4), followed by addition of a pyridoxyl phosphate derivative to lower the oxygen affinity (36). This product has a P_{50} of 20-23 mmHg and an average weight of 150 kDa (range: 64 – 400 kDa) (37). **Hemopure**’s third-generation product, HBOC-201, is derived from bovine hemoglobin, which naturally has a lower oxygen affinity than human hemoglobin. It is also polymerized with glutaraldehyde (38). This product has the highest P_{50} of all HBOCs tested in clinical trials ($P_{50} = 40$ mm Hg). This is a lower oxygen affinity than that of native RBCs. It also has an increased molecular weight, with an average of 250 kDa (Range: 130-500) (36). There are a number of parallels between these products. Although they both exhibit an increased average molecular weight, the large weight range makes both of these products highly heterogeneous. Even if the average molecular weight is ~250 kDa, the smaller species in the heterogeneous mixture are still able to extravasate and scavenge NO. In addition, both materials have low oxygen affinities and low Hill coefficients (n_{50}), limiting their ability to deliver oxygen safely and effectively based on our current state of knowledge on the subject (38).

A recent hypothesis is that size is not the sole determinant of the propensity of the protein to extravasate into the interstitial spaces of the endothelium. Instead, it was proposed that attractive forces control the rate of extravasation (39). While PEGylation

can produce external electroneutrality, surface modification with glutathione (40) produces a net negative charge, causing the protein to be repelled from the vicinity of the endothelium. HemoBioTech's product **HemoTech** is based on this notion and consists of bovine hemoglobin cross-linked with adenosine 5'-triphosphate and surface "decorated" with *o*-adenosine (from reaction of adenosine with periodate) and reduced glutathione (40). According to corporate-produced literature, glutathione reduces the potential oxidative stress inherent in HBOCs (41) while adenosine provides anti-inflammatory activity and contributes to vasorelaxation (42). It is not clear that these would be achieved in circulation since no evidence is yet available to support the claims.

Protein-engineered hemoglobin. The production of recombinant hemoglobin from bacterial expression was pioneered by Sligar (43) and by Olson (44). After clinical failures with DCLHb, Baxter's efforts were directed toward using recombinant techniques to genetically alter heme pocket residues in an attempt to minimize the NO/HBOC interaction. Additionally, fusion of the α -chains into a single chain (α -chain's C-terminal is connected by alanine to β -chain's N-terminal), provided the equivalent of a cross-linked tetramer (45). This led to the creation of **rHb 2.0**, a recombinant Hb shown to have minimal NO/HBOC interaction while maintaining oxygen binding ($P_{50} = 34$ mmHg). However, early Phase II clinical studies indicated that this material had unfavourable properties, leading Baxter to abandon the development of HBOCs (35, 98).

In another approach, Olson and co-workers employ the use of site-directed mutagenesis of key residues involved in ligand binding in the heme pocket and in the $\alpha_1\beta_1$ interfaces gave materials with a reduced affinity for NO (46), modulated oxygen affinity with a high degree of cooperativity (46). However, it still remains a significant challenge to design a material that has a low affinity for NO at a heme site but which binds oxygen effectively for delivery. Hemoglobin mutants with a decreased affinity for NO also showed a decreased affinity for oxygen. The inherent similarity of the two diatomic species limits possible approaches based on alternations of the protein by chemical means (47-49).

Increasing oxygen affinity. Another method of raising molecular mass is to conjugate the protein with inert polymers such as polyethylene glycol (PEG) (Figure 1.4).

MP4OX from Sangart, Inc. is a thiol-mediated, maleimide-PEG-conjugated hemoglobin (69, 102). The 95 kDa species has a very high oxygen affinity ($P_{50} = 5$) that limits release of oxygen in arterioles (23). The high affinity for oxygen comes from PEGylation at sites that are close to the $\alpha_1\beta_2$ interface, an effect that disrupts the T $\rightarrow$ R state transition (51). This alters the equilibrium between the two conformations, stabilizing the high affinity R-state. Although the initial rationale for the conjugation of PEG onto hemoglobin was to increase its size and hydrodynamic volume, the researchers believe that a favourable side effect of PEG conjugation at β -Cys93 is the increased oxygen affinity as a result of R-state stabilization (22). It was proposed that **MP4OX** will prevent premature off-loading of hemoglobin. Recent clinical reports suggest that the typical HBOC side effects of hypertension, which were notable with other products at similar doses, were not observed with infusions of **MP4OX** (50). The authors attribute this to the maintenance of capillary perfusion in combination with targeted oxygen transport (50).

1.5 Future Prospects: Understanding HBOC-induced Vasoconstriction

The setbacks in the development of HBOCs have prompted researchers to elucidate fully the mechanisms of toxicity and to find strategies to reduce toxicity. In reviewing the existing information about the characteristics and clinical profiles of the products that are or were in development, certain requirements for a way forward stand out. With respect to physical properties, it is an accepted theory that increased molecular size serves to prevent extravasation into spaces of high NO concentrations (24). However, the existing method of increasing size leads to heterogeneous mixtures of products characterized by variable structural and functional properties. Commercial use of an approved HBOC must be injected in quantities of the order of 100 g/L, and even 0.1 % impurity can trigger unwanted reactions (17). This implies that homogeneity is a critical issue and hemoglobin derivatives must be produced to give pure, well-defined chemical preparations.

As well, in order to appreciate the complexities of HBOCs and to understand how a patient's organ and whole-body physiology adjust to hypoxia (low oxygen levels), regulation of oxygen transport and hypoxic vasodilation must be fully understood and

controlled (35). Efficient oxygen delivery is governed by an HBOC's ability to acquire oxygen in the lungs and release oxygen at tissue sites. RBCs in whole blood have a P_{50} of 26-31 torr (9). It is now understood that the small size of hemoglobin ($\sim 1/200,000,000$ of RBC) acts more as stepping stones for oxygen off-loading from RBCs, and serves to facilitate oxygen off-loading (52). Thus, an HBOC with low oxygen affinity will release oxygen in the arterioles and capillaries, possibly triggering adverse homeostatic vasoconstrictive responses (50). Alternatively, hemoglobin with a high oxygen affinity will facilitate oxygen release from RBCs in areas of low oxygen partial pressure (18). Nonetheless, the HBOC's affinity for oxygen cannot be so high that it hinders oxygen release. Oxygen delivery within the physiological range also depends strongly on the sigmoidal nature of oxygen binding. Hemoglobin's unique allosteric binding and releasing of oxygen in a sigmoidal fashion is critical for correct oxygenation (9). As a result, an HBOC with good cooperativity will have much better physiological oxygen-carrying ability. An HBOC with poor cooperativity is not expected to deliver oxygen from the lungs to tissue cells efficiently.

Hemoglobin's oxidative and nitrosative reactions are now also believed to have a direct role in HBOC-induced toxicity. Hypoxic vasodilation is a highly conserved physiological vasodilatory response to low tissue oxygen tension that ensures delivery of blood to match metabolic demand (53). Factors that interfere with hypoxic vasodilation thus alter the balance between hypertension and vasodilation. Hypoxic vasodilation is now believed to come from a combination of different sources. These include the allosteric release of NO by sulfhydryl-linked NO (-SNO) (54) that occurs as the RBC deoxygenates, partially oxygenated hemoglobin acting as a nitrite reductase (16, 55), and the metabolic autoregulation of blood flow (22, 50). In addition, the release and binding of ATP to receptors on the endothelium has been shown to stimulate the synthesis of NO by endothelial NO synthase, resulting in increased circulating NO and vasodilation (56). Methods that deal with adverse oxidative and nitrosative effects include adding reducing groups such as glutathione on the surface of the protein (40) to mitigate oxidative stress and chemical additions of adenosine triphosphate (ATP) groups to the surface that serve to elicit signalling pathways for vasodilation through the interaction with receptors (56). As well, chemical alterations that enhance the nitrite reductase activity and increase the

rate of hemoglobin-catalyzed NO production also serve to counteract HBOC-induced vasoconstriction.

In the research reported in this thesis, we first review the chemical biology of nitrite reactions with hemoglobin in Section 1.6 (57). We address the effects of the nitrite reductase activity of modified hemoglobin as a possible vascular source of bioactive NO in Chapter 2 & 3. We then combine many of the features of an ideal HBOC that deal with toxicity issues to produce a novel potential HBOC that we test in animal models (Chapter 4).

1.6 ChemBioChem Review: Reviving Artificial Blood: Meeting the Challenge of Dealing with NO Scavenging by Hemoglobin

Note: The abbreviations for the modified hemoglobin in the publication are different from that in the thesis – Hb-2PEG = Hb-PEG₂, Hb-6PEG = Hb-PEG₆, BT-Hb-6PEG = BT-Hb-PEG₂

1.7 Purpose of Thesis

In order to develop a safe, functional HBOC, the mechanisms of HBOC-induced vasoconstriction should be elucidated. Given the extensive experimental evidence that demonstrates the potential importance of hemoglobin's nitrite reductase activity in mitigating vasoconstriction, we assessed the effects of protein modification on the rate of nitrite reduction. In one example, we compared in detail the difference in reactivity between $\alpha\alpha$ -cross-linked hemoglobin (**DCLHb**) and a second-generation PEGylated hemoglobin (**MP4OX**). Specifically, nitrite-heme reaction rates were used to assess the overall effects of chemical modification on the nitrite reductase activity of hemoglobin. A method for the deconvolution of spectral kinetic data by multi-linear regression analysis was developed in order to obtain rate constants in the kinetic study. In this manner, the specific chemical alterations that enhance NO production *via* nitrite reduction catalyzed by hemoglobin were determined. This work revealed that PEG-hemoglobins convert nitrite to NO at a faster rate than does the native protein (Chapter 2). Since NO scavenging by deoxyhemes is the main cause of HBOC-induced vasoconstriction, hemoglobin derivatives that produce NO at a faster rate through nitrite reduction may serve to compensate for the scavenged NO, leading to decreased vasoactivity.

In considering the extensive work carried out in the search for safe and functional HBOCs, we deduced some logical objectives. The ideal HBOC would have an increased molecular size to prevent extravasation and high oxygen affinity targeting oxygen delivery to capillary beds. In addition, it should have good cooperativity that maintains useful release of oxygen and an increased rate of production of NO from heme-nitrite reactions that can counteract NO scavenging. We addressed all of these factors by producing PEGylated bis-tetramers of hemoglobin (BT-PEG). The chemically enlarged BT-PEG is cross-linked as well as connected between two cross-linked tetramers in addition to PEG conjugation (Chapter 3). BT-PEG is a homogeneous product, an important feature for precise evaluation in animal and potential clinical studies. In depth analytical characterization of PEG-BTs was carried out, and we demonstrated that they appear to satisfy the requirements for an ideal HBOC.

To further test if BT-PEG is functional and efficient in its oxygen delivery *in vivo* while being otherwise physiologically inert, we conducted animal studies of the material in circulation (Chapter 4). The safety of this material in a clinical setting was assessed with blood pressure measurements in both healthy wild-type mice, as well as in obese db/db mice that are highly sensitive to NO scavenging.

Chapter 2 : PEG-Hemoglobins and Nitrite Reductase Activity

2.1 Introduction

The production of NO as catalyzed by deoxyhemoglobin may be a contributing factor in hypoxic vasodilation. The maximal rate of nitrite reduction is reported to be linked to the allosteric structural transition of the hemoglobin tetramer from the oxygenated conformation (R state) to the deoxygenated conformation (T state) (58). It is clear that the nitrite-reductase activity is affected by covalent modifications to hemoglobin. We sought to correlate the change in heme-nitrite reactivity of various modified hemoglobins with their reported clinical vasoactivity. In particular, Winslow and co-workers made the promising discovery that vasoactivity is minimized in a chemically modified hemoglobin containing multiple chains of PEG (**MP40X**) (59) (Scheme 2.1). The decreased hypertensive effect of these PEG-hemoglobins was attributed to their increased size that prevents extravasation and to their high oxygen affinities that would not elicit a homeostatic response by releasing oxygen prematurely in circulation.

Scheme 2.1: Typical methods to conjugate inert polymers onto the surface of hemoglobin include the use of maleimide-activated polyethylene glycol (mal-PEG). Mal-PEG reacts with hemoglobin at the thiol of each β -Cys93 to produce Hb-2PEG. Additional PEG chains can be added by reacting hemoglobin with 2-iminothiolane,

converting lysine amino groups to thiol-containing chains and thus producing additional sites to give Hb-6PEG.

We suggested that the decreased pressor effect exhibited by PEG-hemoglobins could also be the result of enhanced nitrite reductase activity. If the addition of PEG to hemoglobin leads to an increased rate of reduction of endogenous nitrite to produce NO, the modification would serve to counteract NO scavenging by hemoglobin. The enhanced nitrite reductase activity may therefore serve as one of the contributing factors for the PEG-hemoglobin's decreased hypertensive effect as it compensates for removal of NO by hemoglobin.

2.2 Biochemistry (ACS) Article: Polyethylene Glycol Conjugation Enhances the Nitrite Reductase Activity of Native and Cross-Linked Hemoglobin

Note: The abbreviations for the modified hemoglobin in the publication are different from that in the thesis – Hb-2PEG = Hb-PEG5K₂, αα-2PEG = αα-Hb-PEG5K₂, Hb-6PEG = Hb-PEG5K₆.

2.3 Detailed Experimental Methods (60)

2.2.1 Chemical Modification of Hemoglobin: Cross-linking and PEGylation

Cross-linking. Hemoglobin was cross-linked between α -subunits as a bis-fumaryl amide of the α -amino groups derived from the side chains of Lys-99. Our procedure followed that reported by Walder and coworkers (11). A solution of COHb (11 mL, 5.65×10^{-6} mol) in 0.05 M Bis-Tris buffer, pH 7.2 was converted to oxyHb under a stream of oxygen at 0°C with tungsten-lamp irradiation and stirring for two hours. Five equivalents of inositol hexaphosphate (35.6 μ L, 3.75×10^{-4} mol) were added to the solution and the mixture placed under a stream of humidified nitrogen for 2 hours at 37°C. Inositol hexaphosphate was added to block the bis-phosphoglycerate (BPG) binding site, forcing the small linker to site-specifically react within the $\alpha\alpha'$ -subunit. Four equivalents of solid 3,5-dibromosalicyl fumarate (0.0152g, 2.26×10^{-5} mol) was added and the solution stirred for 18 hours (Scheme 2.2). Excess glycine was then added to the mixture to destroy excess reagent. The mixture was cooled in ice and placed under a stream of carbon monoxide for 10 min then passed through a column of Sephadex G-25 that had been equilibrated with MOPS buffer (0.1 M pH 7.2) to remove excess reagent. The cross-linked hemoglobin product ($\alpha\alpha$ -Hb) was concentrated through a membrane with centrifugation (3000 rpm for 30 min), flushed with carbon monoxide, and stored at 4 °C.

Scheme 2.2: DBSF cross-linking of hemoglobin to produce $\alpha\alpha$ -Hb. Inositol hexaphosphate blocks the BPG binding site, forcing the small linker to site-specifically react within the $\alpha\alpha'$ -subunit.

Conjugation with Polyethylene Glycol. Native hemoglobin and $\alpha\alpha$ -cross-linked hemoglobin (10 mL, 0.5 mM) were separately converted to analogous PEG derivatives. *O*-(2-Maleimidoethyl)-*O'*-methylpolyethylene glycol-(5'000) (Mal-PEG5K) was purchased from Fluka. The protein solution was combined with 10 equivalents (0.25 g, 50 μ mol) of Mal-PEG5K in sodium phosphate buffer (0.1 M, pH 7.4) and kept stirring at 4°C overnight. Since each hemoglobin tetramer has two sulfhydryl groups that are reactive to Mal-PEG5K, the effective ratio of reagents is 5 equivalents of Mal-PEG5K to one sulfhydryl group. The vials of hemoglobin solutions were kept under carbon monoxide to prevent possible heme auto-oxidation. PEGylation occurs at β -Cys93, giving products with two PEG chains per tetramer: Hb-2PEG and $\alpha\alpha$ -Hb-2PEG (Scheme 2.3). A species with six PEG chains conjugated to hemoglobin (Hb-6PEG) was prepared as described by Manjula et. al (Scheme 2.3). (61). Ten equivalents of 2-iminothiolane (6.88 mg, 50 μ mol) and 20 equivalents of Mal-PEG5K (0.5 g, 0.1 mmol) were added to the hemoglobin mixture in a single step. The reaction mixture was stirred for 16-20 hours at 4°C. The resulting mixtures were passed through a column containing Sephadex G-25 equilibrated with sodium phosphate buffer (0.1 M, pH 7.4), concentrated and stored at 4°C. Separation from excess reagents was carried out by dialysis in phosphate buffer, (I = 0.1 M, pH 7.4) at 4°C. The buffer was replaced three times, at 12 hr intervals. The PEG-conjugated hemoglobins were then removed and the solution treated with carbon monoxide to produce the carbonmonoxy derivatives. These were stored at 4°C.

Scheme 2.3: (A) Conjugation of mal-PEG5K to a thiol at β -Cys93 of hemoglobin (Hb-2PEG). (B) Cyclic 2-iminothiolane reacts with ϵ -amino groups of lysines to produce an extended thiol that reacts with free Mal-PEG5K. Conjugation of PEG to β -Cys93 of the same hemoglobin also occurs, generating a hexa-PEG conjugate (Hb-6PEG).

2.2.2 Analytical Methods: HPLC, SDS-PAGE, CD spectroscopy, Oxygen Binding

HPLC Analysis. Cross-linked hemoglobins were analyzed using analytical reverse-phase HPLC with a 330 Å C-4 Vydac column (4.6 x 250 mm) to determine the sites of globin chain modifications. Modified and unmodified globin chains were separated using an eluting solvent containing 0.1 % trifluoroacetic acid and a gradient beginning with 20% and ending with 60 % acetonitrile (v/v %) in water (Full procedure in **Appendix 2.1**). The effluent was monitored at 220 nm. PEG-conjugated hemoglobins were individually analyzed using a preparative size-exclusion column: Superdex G-200 HR (10 x 300 mm). Protein samples (0.5 mM) were eluted under partially dissociating conditions by the addition of 0.5 M magnesium chloride in buffer (25 x 10⁻³ M Tris-HCl, pH 7.4). The effluent was monitored at 280 nm.

SDS-PAGE Analysis. Protein standards, reaction samples, and native Hb were

prepared by combining 2-4 μL with the loading buffer (16-18 μL), consisting of 0.0625 M Tris-HCl (pH 6.8), 1.3 M glycerol, 2% SDS, 0.0125 (w/v) bromophenol blue and 0.7 M β -mercaptoethanol. The samples were denatured by heating at 95°C for 10 min. Then, a 7 μL sample was loaded onto a polyacrylamide slab (12% Tris-HCl). The gel was processed in a dual-slab cell apparatus at 200 mV in 0.12 M Tris, 1 M glycine, and 0.014 M SDS running buffer. The gels were stained with Coomassie Brilliant Blue R-250, then de-stained with 30% methanol-10% acetic acid solution.

CD Spectroscopy. In order to determine the effects of PEG modification on the overall structural stability of the protein, we compared the CD spectra of native hemoglobin with that of the PEG-conjugated materials. Protein samples (5.0 μM heme) were prepared in 0.01 M phosphate buffer ($I = 0.02$ M, pH 7.4) and pipetted into cylindrical quartz cuvettes with light path length of 20 mm and volume of 5.7 mL. The CD spectrum from 200-260 nm is obtained in triplicates (Full procedure in **Appendix 2.2**).

Thiol determination with 5,5'-dithiobis(2-nitrobenzoate) (DTNB). Accessible sulfhydryl groups were quantified by observing the results of the disulfide exchange reaction of the β -Cys93 thiols and DTNB at 412 nm. The conditions for the DTNB titration were as follows, 50 mM Bis-Tris, pH 7.4, 0.5 mM EDTA, 25 mg/mL protein and 4 mM DTNB. We produced a standard curve using known concentrations of β -mercaptoethanol. Thus, spectroscopic measurements of the DTNB reaction with modified hemoglobins provided the residual thiol concentration after subtraction of background absorbance (Full procedure in **Appendix 2.3**).

2.2.3 Kinetic Measurements

Solutions containing $\alpha\alpha$ -Hb, $\alpha\alpha$ -Hb-2PEG, Hb-2PEG, or Hb-6PEG in buffers other than bis-tris were exchanged for bis-tris (0.01 M, pH = 7.2). The concentrations of hemoglobin solutions were determined using the cyano-methemoglobin method (62). In a 10 mL two-neck round bottom flask, hemoglobin solutions were adjusted to 0.03-0.05 mM with dilutions of bis-tris buffer (0.01 M, pH = 7.2) in a 1.5 mL volume. This solution was oxygenated for 2 hrs, 0°C, and thoroughly deoxygenated for 2 hrs, 37°C, by flushing

with nitrogen. As well, oxygen-free stock solutions of nitrite (100 mM or 10 mM) and a sealed cuvette were deoxygenated by flushing with nitrogen for 2 hrs during the hemoglobin deoxygenation time period. Deoxyhemoglobin was then transferred to the sealed cuvette (that has been flushed with nitrogen) using a gas-tight syringe. This syringe was purged with nitrogen prior to the solution transfer. Once the hemoglobin was added into the sealed cuvette, the anaerobic solutions of nitrite were added to give final concentrations of nitrite of 0.05-1.5 mM (Full procedure in **Appendix 2.4**). The formation of methemoglobin (MetHb) and iron nitrosyl hemoglobin (HbNO) were followed by recording spectra from 500 to 650 nm. Spectral data were analyzed by multiple linear regression analysis of data at one nm intervals using separately obtained spectra of the individual components as a basis set. The initial reaction rate is the rate of ferric heme formation at the beginning of the reaction calculated as the average rate over the first 100 seconds.

2.2.4 Deconvolution of Spectra

Using known concentrations of pure samples of deoxyHb, oxyHb, metHb and NOHb, the respective molar extinction coefficients (ϵ_{deoxy} , ϵ_{oxy} , ϵ_{met} , ϵ_{NOHb}) can be calculated using the Beer-Lambert law. Pure metHb is prepared by reacting hemoglobin with potassium ferricyanide (5 equivalents to 1 heme), and pure NOHb is prepared using an *in-situ* method of generating NO in solution. Excess sodium dithionite is added to deoxygenated hemoglobin and a stock solution of nitrite is added to the deoxyHb sample (63). The nitrite is reduced by dithionite to form NO, which binds quickly to the hemoglobin, allowing for stoichiometric amounts of NOHb. These baseline spectrums can then be incorporated into the deconvolutional analysis as a reference spectrum.

Deconvolution of the reaction progress over time can be done using the Beer-Lambert law. The absorbance of the reaction (Abs_{rxn}) between deoxyhemoglobin and nitrite at any give time over the course of the reaction is a component of all the different species of hemoglobin present (deoxyHb, oxyHb, metHb, NOHb), as shown in the following equation:

$$\text{Abs}_{\text{rxn}} = \epsilon_{\text{deoxy}} c_{\text{deoxy}} l + \epsilon_{\text{oxy}} c_{\text{oxy}} l + \epsilon_{\text{met}} c_{\text{met}} l + \epsilon_{\text{NOHb}} c_{\text{NoHb}} l$$

Fitting of the reference spectra using molar extinction coefficients (ϵ_{deoxy} , ϵ_{oxy} , ϵ_{met} , ϵ_{NOHb}) to the reaction spectra was then carried out using R.cran using a least-squares method (Full procedure in **Appendix 2.5**):

$$\Sigma((\epsilon_{\text{deoxy}}c_{\text{deoxy}}l + \epsilon_{\text{oxy}}c_{\text{oxy}}l + \epsilon_{\text{met}}c_{\text{met}}l + \epsilon_{\text{NOHb}}c_{\text{NOHb}}l) - \text{Abs}_{\text{rxn}})^2 = L$$

2.4 Supplementary Information

2.4.2 CD Spectroscopy

The CD spectrum (200-260 nm, indicative of secondary structure change), of all cross-linked and PEG conjugated hemoglobins were identical to that of native hemoglobin (Figure 2.1). Although protein side chains had been modified, no disturbances to the tertiary structure of the protein are likely to have occurred.

Figure 2.1: CD spectra (200-260 nm) of modified hemoglobins compared to native hemoglobin (solid line). No significant alterations to the protein secondary structure were observed after protein cross-linking or PEGylation.

2.4.2 Kinetic Measurements: Nitrite Reductase Activity

Deconvolution of spectral data for the reactions of modified hemoglobin derivatives with nitrite yielded individual component concentrations (deoxy, oxy, met and NO) over the course of the reaction. The apparent rate constants for each reaction were determined using initial rate kinetics at constant hemoglobin concentrations and plotted as a function of nitrite concentrations.

Figure 2.2: (A) Kinetic time scans (30s) and (B) deconvoluted data showing progress of the reactions between 0.03 mM $\alpha\alpha$ -Hb with 1.5 mM Nitrite. (C) Initial rate plot as a function of nitrite concentration for $\alpha\alpha$ -Hb.

Figure 2.3: (A) Kinetic time scans (30s) and (B) deconvoluted data showing progress of the reactions between 0.03 mM $\alpha\alpha$ -2PEG with 0.75 mM Nitrite. (C) Initial rate plot as a function of nitrite concentration for $\alpha\alpha$ -2PEG.

Figure 2.4: (A) Kinetic time scans (30s) and (B) deconvoluted data showing progress of the reactions between 0.03 mM Hb-2PEG with 0.25 mM Nitrite. (C) Initial rate plot as a function of nitrite concentration for Hb-2PEG.

Figure 2.5: (A) Kinetic time scans (30s) and (B) deconvoluted data showing progress of the reactions between 0.03 mM Hb-6PEG with 0.5 mM Nitrite. (C) Initial rate plot as a function of nitrite concentration for Hb-6PEG.

2.5 Summary

We have examined the effects of chemical modifications of hemoglobin on its nitrite reductase activity. We follow the rate of methemoglobin formation rather than deoxyhemoglobin consumption since the formation of metHb comes exclusively from the reaction of deoxyHb with nitrite. We observe that all hemoglobins with PEG conjugation at β -Cys93 (native and cross-linked) produce NO at a faster rate than the native protein. It appears that modifications at this key residue are especially important for increased

activity. Further PEGylation at surface lysine residues does not affect this activity. β -Cys93 is located within the $\alpha_1\beta_2$ -interface (64). This similarity is also reflected in their oxygen binding properties; both have low P_{50} and reduced cooperativity. Changes to this residue can alter the equilibrium between the T-state and R-state that favour the R-state. It follows that increased heme-nitrite reactivity is likely to be due to the R-state stabilizing effect of PEG conjugation at β -Cys93 (65). Hemoglobins that are R-state stabilized exhibit a lower heme redox potential and are more easily oxidized. The lower cooperativity of PEG-hemoglobins also maintains the R-conformation, which is another source of the increased rate of reaction. Since these derivatives are in their high ligand binding geometry, this further facilitates the association of nitrite with the heme iron (66). This effect is also reflected in the increased affinity for oxygen exhibited by both Hb-2PEG and Hb-6PEG. The degree to which the protein is in the R-state can typically be correlated with its affinity for oxygen.

The enhanced nitrite reductase activity exhibited by PEG-hemoglobins (Hb-2PEG and Hb-6PEG) correlates well with the reported decreased hypertensive effect in animal studies. This suggests that the reduced vasoactivity reported for PEG-conjugated hemoglobins, such as Sangart's **MP4OX** product (22, 50) (Hb-6PEG), could be the result of increased nitrite reductase activity. These experimental results lend support to the theory that nitrite can be a vascular bioactive precursor pool for NO production.

Chapter 3 : Enhancing Nitrite Reductase Activity – PEGylated Bis-Tetramers

3.1 Introduction

Setbacks in the search for safe and functional HBOCs have prompted researchers to find new strategies that alleviate the toxicities associated with HBOC administration. It follows that an HBOC with an increased molecular size, high affinity and cooperativity for oxygen and enhanced nitrite reductase activity will have some of the necessary properties for a safe blood substitute. We have now shown that the addition of PEG to hemoglobin at β -Cys93 significantly increases the rate of reduction of nitrite to NO compared to native and cross-linked hemoglobin (60). The increased rate of NO production may therefore also contribute to the decreased hypertensive effect observed with larger PEGylated hemoglobins (39, 55, 58, 67, 68).

However, although PEG-hemoglobins have favorable properties (increased size and NO production) the PEG-conjugated tetramer derivatives are not cross-linked with the PEG chains attached at the protein surface of single subunits. These materials exhibit a propensity to dissociate into PEGylated dimers (51). The dissociated dimers will not be effective as oxygen carriers and are potentially harmful. In addition, the extremely high oxygen affinity of the PEGylated tetramers combined with their low cooperativity ($P_{50} = 3.6$, $n_{50} = 1.8$) are viewed to be potentially problematic in their oxygen delivery (51). Although a high affinity for oxygen is now seen as a favorable quality, (50, 69), it must be associated with high cooperativity in order to permit oxygen delivery within the physiological range. (70).

An alternative method that produces larger entities while retaining cooperativity comes from efficient chemical procedures that produce cross-linked hemoglobin bis-tetramers (BT-Hb). This has previously been achieved in our lab (71-73). Tetra-functional cross-linkers react selectively within hemoglobin tetramers while also creating inter-protein linkages between tetramers (71-73) to give cross-linked bis-tetramers of

hemoglobin (BT-Hb) (Scheme 3.1). This process differs from traditional methods that polymerize hemoglobin tetramers with the non-specific reagent, glutaraldehyde (38, 50). The variability in composition and structure of such heterogeneous mixtures made it difficult to correlate the infusion of HBOCs with clinical events whereas the structurally defined bis-tetramers will give specific information.

BT-Hbs are homogenous, high molecular weight compounds with increased size, altered geometry and oxygen carrying sites that are present in the same proportion as those in native hemoglobin. The high purity of these compounds comes from the specific acylation reactions within the BPG binding site of the protein. The di-bromosalicyl (DBS) leaving groups of the cross-linker have negative charges that direct reaction specifically to the cationic BPG site, where the lysine-82 can react with electrophilic ester groups to form stable amide bonds (71, 74). This site-specific cross-linking overcomes problems with heterogeneity and eliminates the presence of dissociated $\alpha\beta$ -dimers (71, 74). In addition, allosteric cooperativity is retained due to tetrameric stabilization and the inter-protein linkage effectively doubles the size of the hemoglobin molecule. It follows that PEGylation of such entities will not only further increase the size of the protein assembly, it will also alter the oxygen affinity (lower P_{50}) and enhance the nitrite-reductase activity (74). We have now examined the nitrite reductase properties of BT-Hb derivatives as well as their PEG-conjugates (BT-PEG).

Scheme 3.1: Bis-tetramers of hemoglobin (BT-Hb) with linkages between protein subunits. Reaction with maleimide-PEG at β -Cys93 produces PEGylated bis-tetramers of hemoglobin (BT-PEG).

We report that the enhanced rate of NO production associated with PEGylation at β -Cys93 is also observed with BT-PEG. We find that the rate of nitrite reduction correlates with the protein's affinity and cooperativity for oxygen. These materials have properties that one would expect to be important for a novel HBOC that is both safe and effective for oxygen delivery and transport in circulation.

2.2 Biochemistry (ACS) Article: Enhancing Nitrite Reductase Activity of Modified Hemoglobin: Bis-tetramers and Their PEGylated Derivatives

Note: The abbreviations for the modified hemoglobin in the publication are different from that in the thesis –BT-PEG = BT-Hb-PEG5K₄.

3.3 Detailed Experimental Methods (74)

3.3.1 General Methods

The tetra-functional reagent *N,N'*-Bis(bis(3,5-dibromosalicyl)isophthalyl)-5,5'-sulfonyl bis(1,4-(phenylenecarbonylimino))bis-1,3-benzenedicarboxylate (Reagent **1**) was synthesized according to Hu. et. al. (Figure 3.1) (75).

Figure 3.1: Cross-linking reagent (**1**) used in reactions with hemoglobin to produce bis-tetramers.

3.3.2 Chemical Modification of Hemoglobin: Cross-linking and PEGylation

Cross-linking. Bis-tetramers of hemoglobin (BT-Hb) were produced through the reaction of the tetrameric acylating reagent **1** with native unmodified hemoglobin. The concentration of hemoglobin tetramers was maintained at 0.5 mmol/L for all cross-linking reactions. A solution of carboxyHb in sodium borate buffer (0.05 M, pH 7.4) was oxygenated under a stream of oxygen at 0 °C with tungsten-lamp irradiation and stirring for two hours before deoxygenation under a stream of humidified nitrogen for two hours at 37°C. Since reagent **1** is not readily soluble in aqueous media, DMSO was used to dissolve the reagent. The concentration of DMSO in all reactions did not exceed 2.5 %. The dissolved reagent in DMSO is then added to the deoxy-Hb to make the final

concentrations of Hb 0.5 mM and reagent **1** 1.0 mM. The reaction was then allowed to proceed for 18 hours under a stream of humidified nitrogen at 37 °C to ensure full deoxygenation during the cross-linking reaction. The mixture was cooled on ice and placed under a stream of carbon monoxide for 10 min then passed through a column of Sephadex G-25 equilibrated with MOPS buffer (0.1 M, pH 7.2) to remove excess reagent. The cross-linked hemoglobin product was concentrated through a membrane with centrifugation (3000 rpm for 30 min) and stored at 4°C until further purification.

Isolation of Modified Hemoglobins. Purification of BT-Hb was carried out using gel-filtration chromatography (Sephadex G-100, 1000 x 35 mm) under slightly dissociating conditions. The eluent used was 25 mM Tris-HCl, pH 7.4, containing 0.5 M magnesium chloride. The high salt concentration causes partially modified hemoglobin tetramers to dissociate, allowing for separation of larger bis-tetramers from the mixture. Fractions were collected, concentrated and analyzed using Superdex G-200 size-exclusion chromatography, c4 reverse-phase analytical HPLC, and SDS-PAGE gel electrophoresis. The fractions with pure bis-tetramers were pooled together and exchanged into either sodium phosphate buffer (0.1 M, pH 7.4) for further PEGylation, or into bis-tris (0.01M, pH 7.2) for kinetic studies.

Conjugation with polyethylene glycol (PEG). Protein solutions of purified BT-Hb (10 mL, 0.5 mM) were combined with 10 eq of methoxypolyethylene-glycol-(5000)-maleimide (Mal-PEG5K) in sodium phosphate buffer (0.1 M, pH 7.4) and kept at 37 °C overnight to give products with two PEG chains per tetramer: BT-PEG (Scheme 3.2). Although PEGylation is typically carried out at low temperatures (4 °C), it was found that harsher conditions were required for complete reaction. This was because BT is cross-linked at the β -site and resulted in a protein conformation that does not have sulfhydryl residues readily accessible. After complete reaction, the resulting mixture was passed through a Sephadex G-25 column equilibrated with sodium phosphate buffer (0.1 M, pH 7.4), concentrated and stored at 4°C. Separation from excess PEG reagents was carried out by dialysis in phosphate buffer, ($I = 0.1$ M, pH 7.4) at 4°C. The buffer was replaced three times, at 12 hr intervals. The PEG-conjugated hemoglobins were then removed and the solution treated with carbon monoxide to produce the carbonmonoxy derivatives. These were stored at 4°C.

Scheme 3.2: Reaction of purified BT-Hb with maleimide-active PEG results in complete PEGylation at β -Cys-93.

3.3.3 Analytical Methods: HPLC, SDS-PAGE, CD spectroscopy, Oxygen Binding

HPLC analysis of modified hemoglobins. Hemoglobins bis-tetramers were analyzed using analytical reverse-phase HPLC with a 330 Å C-4 Vydac column (4.6 x 250 mm) to determine the sites of globin chain modifications (76). Modified and unmodified globin chains were separated using an eluting solvent containing 0.1 % trifluoroacetic acid and a gradient beginning with 20% and ending with 60 % acetonitrile (v/v %) in water (77). The effluent was monitored at 220 nm. PEG-conjugated bis-tetramers (BT-PEG) were analyzed using a Superdex G-200 HR (10 x 300 mm) preparative size-exclusion column. Protein samples (0.5 mM) were eluted under partially dissociating conditions by the addition of 0.5 M magnesium chloride in buffer (25×10^{-3} M Tris-HCl, pH 7.4). The effluent was monitored at 280 nm.

SDS-PAGE analysis. Protein standards, reaction samples, and native Hb were prepared by combining 2-4 μ L with the loading buffer (16-18 μ L), consisting of 0.0625

M tris-HCl (pH 6.8), 1.3 M glycerol, 2% SDS, 0.0125 (w/v) bromophenol blue and 0.7 M β -mercaptoethanol. The samples were denatured by heating at 95°C for 10 min. Then, a 7 μ L sample was loaded onto a polyacrylamide slab (12% Tris-HCl). The gel was processed in a dual-slab cell apparatus at 200 mV in 0.12 M Tris, 1 M glycine, and 0.014 M SDS running buffer. The gels were stained with Coomassie Brilliant Blue R-250, then de-stained with 30% methanol-10% acetic acid solution.

Oxygen Binding Analysis. Oxygen binding measurements (28°C) were measured with a Hemox Analyzer that measures the oxygen pressure for half-saturation (P_{50}) and Hill's coefficient of cooperativity at half saturation (n_{50}). Hemoglobin samples, BT-Hb and BT-PEG (~1 g/L), were equilibrated in sodium phosphate buffer ($I = 0.01$ M, pH 7.4) on a Sephadex G-25 column and adjusted to concentrations give 1 mM hemoglobin. Each hemoglobin sample is then oxygenated for 2 hrs at 0 °C under flowing oxygen prior to analysis. The sealed isothermal UV/Vis cell is filled with the oxygenated hemoglobin sample (10 mL) and further oxygenated by flowing air, at 20°C. Recording of the change in absorbance at 560 nm with respect to the oxygen pressure, pO_2 begins when the sample is deoxygenated by flowing nitrogen. The data was then fitted to the Adair equation to obtain P_{50} and n_{50} (6).

The degree of saturation (Y) can be calculating from the following equation:

$$Y = (\text{Abs} - \text{Abs}_{\min}) / (\text{Abs}_{\max} - \text{Abs}_{\min})$$

The oxygen pressure (pO_2) at half saturation ($Y = 0.5$) is the P_{50} and is a point of comparison of the oxygen affinity between different hemoglobin species. The Hill coefficient (n_{50}) represents the degree of cooperativity between the hemes of the hemoglobins, and can similarly be determined as the slope at half saturation of $\log(Y / 1 - Y)$ plotted against $\log(pO_2)$:

$$\log(Y / 1 - Y) = n \log(pO_2) - n \log(P_{50})$$

3.3.4 Kinetic Measurements

Kinetic experiments were conducted in the same manner as described in Lui, et. al. (60) Briefly one mL hemoglobin samples (5-10 μM) in bis-tris (0.01 M, pH 7.2) buffer were thoroughly flushed with nitrogen and the resulting solutions transferred anaerobically using a gas-tight syringe into a sealed cuvette. Oxygen-free solutions of nitrite were then added to give final concentrations of nitrite of 50-200 μM . The formation of methemoglobin (MetHb) and iron nitrosyl hemoglobin (HbNO) were followed by recording spectra from 500 to 650 nm and spectral data were analyzed by multiple linear regression analysis using separately obtained spectra of the individual components as a basis set (65). All kinetic runs were maintained at 25°C with a circulation bath through the jacketed cell compartment of a UV-Vis spectrophotometer. The initial reaction rate is for ferric heme formation as the average rate over the first 100 seconds after mixing.

3.4 Supplementary Information

3.4.1 Kinetic Measurements

Deconvolution of spectral data for the reactions of modified hemoglobin derivatives with nitrite yielded individual component concentrations (deoxy, oxy, met and NO) over the course of the reaction. The apparent rate constants for each reaction were determined using initial rate kinetics at constant hemoglobin concentrations and plotted as a function of nitrite concentrations.

Figure 3.2: (A) Kinetic time scans (30s) and (B) deconvoluted data showing progress of the reactions between 5 μM BT-Hb with 0.4 mM Nitrite. (C) Initial rate plot as a function of nitrite concentration for BT-Hb.

Figure 3.3: (A) Kinetic time scans (30s) and (B) deconvoluted data showing progress of the reactions between 5 μM BT-PEG with 0.2 mM Nitrite. (C) Initial rate plot as a function of nitrite concentration for BT-PEG.

3.5 Summary

A primary goal in the development of novel HBOCs is to be able to correlate properties of altered hemoglobins with their physiological effects in animal models. Homogeneous solutions of hemoglobin bis-tetramers (BT-Hb) can be produced efficiently to give materials with well-defined structural integrity (71). BT-Hb has an increased molecular size while retaining appropriate oxygen affinity and cooperativity. Analysis of its reaction with nitrite reveals that although its heme-nitrite reaction rates are

enhanced ($\sim 3\times$ faster than native hemoglobin), it reacts with rates similar to its cross-linked counterpart ($\alpha\alpha$ -Hb). In contrast, PEGylation at β -Cys93 significantly increases the rate of nitrite reduction to NO, yielding a material with an enhanced nitrite reductase activity, improved oxygen affinity (lower P_{50}) and good cooperativity.

Figure 3.4: Correlation between oxygen affinity of modified hemoglobins and nitrite reductase activities (NiR). **(A)** Poor correlation when cell-free native hemoglobin data is included ($R^2 = 0.345$) **(B)** Good correlation when cell-free native hemoglobin data is removed ($R^2 = 0.845$).

We observe a correlation between the rates of nitrite reduction and the oxygen binding properties of the protein (both allosteric cooperativity and its affinity for oxygen) (57). A general trend is observed that hemoglobin derivatives with higher oxygen affinity (low P_{50}) have faster heme-nitrite reaction rates (Figure 3.4b). However, inclusion of the data for native hemoglobin lowers the coefficient of determination R^2 from 0.845 to 0.345 (Figure 3.4a). Native cell-free hemoglobin in solution at μM concentrations is reported to be significantly dissociated into its dimeric form (17). Dimeric hemoglobin has a much higher affinity for oxygen ($P_{50} \sim 5$ torr) than tetrameric hemoglobin, accounting for the poor correlation between NiR and P_{50} (Figure 3.4a). Omitting the data for dimeric native hemoglobin gives a much better correlation ($R^2 = 0.845$) between the tetrameric forms of hemoglobin (Figure 3.4b). The enhanced rate of nitrite reduction

exhibited by derivatives with higher oxygen affinities is now attributed to come from both a decreased redox potential and an increased ligand binding affinity resulting from the R-state geometry of these proteins (66).

The redox potential (electron transfer propensity) of a chemical species measures its ability to be reduced through the addition of electrons. An increase in redox potential ($E_{\text{cell}} > 0$) is reflective of a spontaneous forward reaction. Conventional redox reactions are written from the oxidized form going to the reduced form: (*e.g.*) $\text{Fe}^{\text{(III)}} \rightarrow \text{Fe}^{\text{(II)}} + \text{e}^-$ (forward reaction). In the reduction of nitrite catalyzed by ferrous heme, the iron centre is oxidized from $\text{Fe}^{\text{(II)}}$ to $\text{Fe}^{\text{(III)}}$ (conventionally backwards). As a result, in order for this reaction to occur more favourably (more spontaneous), a decrease in redox potential will allow for the conventionally ‘backwards’ reaction: $\text{Fe}^{\text{(II)}} + \text{e}^- \rightarrow \text{Fe}^{\text{(III)}}$ to occur more favourably. The influence of modifications at β -Cys93 by *N*-ethylmaleimide (NEM) on the protein’s redox potential has been previously reported (99). NEM-Hb has a lower redox potential ($E_{1/2} = 45 \text{ mV}$) compared to that of native hemoglobin ($E_{1/2} = 85 \text{ mV}$) (99, 100). This further supports the theory that PEGylation at β -Cys93 will result in a similar decrease in redox potential as observed in the case for NEM-modified hemoglobins. While heme redox potentials are important in determining the rate of nitrite reduction, high ligand affinity (heme pocket ligand-binding properties) may also contribute to the increased reactivity (66). R-state hemoglobin will bind oxygen with increase affinity and likewise facilitate nitrite binding, potentially contributing to enhanced formation of NO from nitrite (74).

Figure 3.5: Correlation between oxygen cooperativity of modified hemoglobins and nitrite reductase activities (NiR).

We also observe that a decrease in allosteric cooperativity results in an increased rate of reaction with nitrite. Hemoglobin derivatives with low cooperativity but high oxygen affinity remain in the faster reacting R-state, facilitating the reduction of nitrite to NO. Proteins that are frozen in the R-state geometry not only have a lower redox potential, they bind ligands in a hyperbolic manner rather than in a sigmoidal manner. In contrast, derivatives with a high degree of allosteric cooperativity (a favorable feature for efficient oxygen delivery) but low affinity for oxygen will have lower rates of NO production. It appears that a protein's nitrite reductase activity is a complex reflection of all these factors. Predicting function based on the nature of structural alterations thus requires understanding the relationship between P_{50} , n_{50} and its nitrite reductase activity.

Although BT-PEG is not as efficient as simple PEG-Hbs (Hb-2PEG and Hb-6PEG) in its heme-nitrite reactions, its combination of increased size, non-globular shape and favourable cooperativity would contribute to its being an acceptable candidate to be a safe and effective HBOC in circulation.

Chapter 4 : Physiological Responses of Bis-Tetramers and their PEGylated Derivatives

4.1 Introduction

The nitrite reductase activity of hemoglobin derivatives varies according to the nature of chemical alterations used to modify the protein (60). We have shown that increased rates of NO production from nitrite as catalyzed by hemoglobin correlate well with materials that show decreased vasoactivity in clinical trials (74). While this relationship is apparent in *in vitro* studies, the effect of hemoglobin's nitrite reductase activity *in vivo* has not been previously explored. Here we compare two hemoglobin derivatives, BT-Hb and BT-PEG in an animal model study.

The experimental design for the set-up in pre-clinical animal models is particularly important to predict the outcomes that would be observed in human clinical trials. It turned out that the complications from administration of HBOCs conducted in healthy, inbred young animals do not mimic those of clinical tests. Many of the patients in clinical studies that need blood transfusions have significant underlying health disorders (35). In fact, a prominent feature of these patients is often the presence of endothelial dysfunction, including impaired NO response (78, 79). It is conceivable that the administration of an HBOC into the circulation will exacerbate their impaired NO availability. An animal system with existing endothelial dysfunction will clearly serve as a better model for HBOC-toxicity studies in those cases (35).

Figure 4.1: Bis-tetramers of hemoglobin (BT-Hb) are cross-linked tetramers with an inter-protein linkage. PEGylation at β -cysteine residues produces BT-PEG with four PEG chains attached at the protein surface.

Here we conducted *in vivo* experiments with healthy wild-type (WT) mice as well as diabetic (db/db) mice. Db/db mice have inherent endothelial dysfunction and vascular complications associated with diabetes and are thus highly sensitive to systemic vasoconstriction (80). We examined the systemic hypertensive effects of both BT-Hb and BT-PEG (Figure 4.1) in awake mice with a non-invasive blood pressure system. The effect of these materials on the host animal's blood pressure was determined and compared to baseline injections of phosphate buffer saline (PBS). We observe that both BT and BT-PEG do not elicit the increase in blood pressure associated with most HBOCs in both WT and db/db mice.

4.2 Experimental

4.2.1 General Methods and Animal Protocols

This study was approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital, Boston, Massachusetts (81). All mice were obtained from Jackson Laboratory (Bar Harbor, ME). The animals in the study were 8-10 week old (25-30 g) male C57BL/6J wild-type (WT) mice and B6.Cg-m^{+/+}Lepr^{db}/J (C57BL/6J background) db/db mice.

4.2.2 Preparation of Bis-Tetramers and PEGylated Bis-Tetramers

Hemoglobin derivatives were prepared according to the methods described by Lui et. al. (74). Briefly, native cell-free carboxyhemoglobin (20 mL, 1.5 mM, 0.03 mmol) was exchanged into 0.05 M sodium borate buffer, pH 7.4 by passing through a Sephadex G-25 column into a round bottom flask. This hemoglobin mixture (~0.6 mM) was oxygenated with a stream of oxygen under irradiation with a tungsten lamp at 0°C for 3 hours. Oxyhemoglobin was then deoxygenated with humidified nitrogen at 37°C for 3 hours. The tetra-functional cross-linker, 3,5-tetrabromosalicyl benzylsulfone (TBSS) (0.1569g, 0.09 mmol) was dissolved in 420 µL DMSO and added to the hemoglobin mixture. The reaction was allowed to proceed overnight, up to 24 hours. Upon completion of the reaction, the mixture was flushed vigorously with carbon monoxide for 10 min and the crude mixture passed through a Sephadex G-25 column equilibrated with 0.1M MOPS, pH 8. The hemoglobin was concentrated and stored at 4°C under CO. Purification of bis-tetramers (BT-Hb) was carried out using gel-filtration chromatography (Sephadex G-100, 1000 x 35 mm) under slightly dissociating conditions. The eluent used was 25 mM Tris-HCl, pH 7.4, containing 0.5 M magnesium chloride. The high salt concentration causes partially modified hemoglobin tetramers to dissociate, allowing for separation of larger bis-tetramers from the mixture. Fractions were collected, concentrated and analyzed using Superdex G-200 size-exclusion chromatography, c4 reverse-phase analytical HPLC, and SDS-PAGE gel electrophoresis. Conjugation with polyethylene glycol (PEG) was carried out in a similar method as described by Lui et. al. (74). Protein solutions of purified bis-tetramers of hemoglobin (40 mL, 0.5 mM, 0.02 mmol) were combined with 10 eq of methoxypolyethylene-glycol-(5000)-maleimide (Mal-PEG5K, 1g, 0.2 mmol) in sodium phosphate buffer (0.1 M, pH 7.4) and kept at 37°C overnight to give products with two PEG chains per tetramer: BT-PEG. The resulting mixture was passed through a Sephadex G-25 column equilibrated with sodium phosphate buffer (0.1 M, pH 7.4), concentrated and stored at 4°C. Separation from excess PEG reagents was carried out by dialysis in phosphate buffer, (I = 0.1 M, pH 7.4) at 4°C. The buffer was replaced three times, at 12 hr intervals. The PEG-conjugated hemoglobins were then removed, the solutions treated with carbon monoxide to produce the

carbonmonoxy derivatives, and stored at 4°C.

The hemoglobin derivatives were then exchanged into a modified PBS solution (0.33g/dL *N*-acetyl cysteine, pH 7.1) using a sephadex G-25 column. The antioxidant *N*-acetyl cysteine was added to the buffer in an effort to minimize rapid auto-oxidation of ferrous heme to ferric heme and its concentration adjusted to be in a 6-fold ratio to hemoglobin. The final concentration of both BT-Hb and BT-PEG were adjusted to be 4 g/dL. The hemoglobin samples were then oxygenated as according to the method previously described, bulk-filtered with a 0.45- μ m filter followed by two sequential 0.2- μ m sterile filtration steps and stored frozen at -20°C.

4.2.3 Preparation of Murine Tetrameric Hemoglobin Solution

Whole blood was obtained from WT mice through puncture of the left ventricle into a heparinized syringe (81). The collected blood was diluted with phosphate-buffered saline (PBS) in a 1:2 ratio and subject to a freeze-thaw cycle (-80°C to room temperature) three times in order to lyse the cells. The mixture was centrifuged at 21,600 g for 1.5 h at 4°C to separate hemoglobin from the cell debris. The supernatant was collected and filtered through a 0.2 μ m Nalgene filter, (Rochester, NY) and dialyzed against a deoxygenated 0.9 % saline solution overnight at 4°C. After purification with dialysis, the solution is then concentrated using a filter (3,000 MWCO Centricon, Centrifugal Filter, Fisher Scientific, Suwanee, GA) and then adjusted to 4 g/dL.

4.2.4 Measurement of Systolic Blood Pressure in Awake Mice

Systolic blood pressure (SBP) was measured with a non-invasive blood pressure system (XBP 1000, Kent Scientific, Torrington, CT) in awake mice (82). Briefly, the mouse was placed in a restrainer (Kent Scientific) for 10 minutes, three times to allow the animal to acclimatize to the device until the mice remained comfortable for prolonged periods. The hemoglobin derivatives (0.48 g Hb/kg) were then administered over 1 minute via a tail vein injection to allow for a ~16 % top load infusion (e.g. 0.3 ml in a 25 g mouse). For experiments with db/db mice, the amount of samples infused was kept to be 0.48 g/kg, leading to a higher top-load infusion (0.6 mL in a 50 g mouse). SBP was

measured every 10 minutes for one hour. The HBOCs of study, bis-tetramers of hemoglobin (BT-Hb, 0.48 g/kg) and PEGylated bis-tetramers of hemoglobin (BT-PEG, 0.48 g/kg), were administered at the same concentration. The animal studies also included positive and negative control groups of murine tetrameric hemoglobin (mTet, 0.48 g/kg) and modified PBS (0.33g/dL *N*-acetyl-cysteine, pH 7.1).

4.2.5 Blood and Tissue Sampling

After two hours of injection, the mice were anesthetized with an intraperitoneal injection of Ketamine (120 mg/kg) and Xylazine (4 mg/kg) and whole blood was obtained through open chest cardiac puncture (80). The mouse was then euthanized by cervical dislocation. The blood gas values and oximetry values were then analyzed with a Radiometer ABL800 Flex system. Blood plasma was obtained by centrifuging the collected whole blood at 4,000 *rpm* for 8 min at 4°C and stored at -80°C. Hb and methHb concentrations of whole blood and plasma were determined by the cyanomethemoglobin method measuring absorption at 540 nm and 630 nm with a spectrophotometer (SepetraMax M5, Molecular Devices).

4.2.6 Statistical Analysis

All data are expressed as mean $\pm$ SD (standard deviation). Data of SBP and HR in awake mice were analyzed by a repeated measures two-way ANOVA with interaction (SigmaStat 3.0.1; Systat Software, Inc., San Jose, CA). A multi-linear regression model analysis was tested in the invasive hemodynamic measurements in anesthetized mice. Group comparison analysis was carried out using MATLAB (r2010b, The MathWorks) to perform the Student *t* test after analysis of variance for plasma methHb levels. A value of $P < 0.05$ was considered significant. All data are expressed as mean $\pm$ SD. Group comparison analysis was carried out using MATLAB (r2010b, The MathWorks) to perform the Student *t* test after analysis of variance. A value of $P < 0.05$ was considered significant.

4.3 Results

4.3.1 Preparation of Hemoglobin Derivatives

BT-Hb and BT-PEG were prepared according to the methods described in Lui et. al. (74). Their chemical and physical properties are described in Table 4.1. After protein modification, the hemoglobin derivatives were transferred into a modified PBS buffer containing the anti-oxidant *N*-acetylcysteine (NAC). NAC was added to the buffer in an attempt to minimize auto-oxidation of the ferrous heme to ferric heme (metHb). The protein concentration used for animal infusion was adjusted to 4 g/dL, which differs from HBOCs of polymerized hemoglobin such as HBOC-201 (Hemopure, 12–14 g/dL) and PolyHeme, (9-10 g/dl). This particular concentration was chosen because the PEGylated HBOC derivative – MP4, is administered at 4 g/dL.

Table 4.1: Chemical and Physical Properties of BT-Hb and BT-PEG

	BT-Hb	BT-PEG
Source	Human	Human
Modification	Cross-linked Inter-protein linked	Cross-linked Inter-protein linked PEGylated
Buffer	Modified PBS(NAC)	Modified PBS(NAC)
pH	7.1	7.1
ctHb (g/dL)	4.0 ± 0.1	4.0 ± 0.1
% Tetramer	0	0
P_{50} (mmHg)	9.3	4.1
n_{50}	2.7	2.4
P_{metHb} (%)	20.2	24.1

Because of the extensive purification procedure to obtain pure bis-tetramers of hemoglobin, there were no unmodified tetrameric species present in the final product that was used in animal testing (Figure 4.2). The chemical reaction to produce bis-tetramers is subject to a competing hydrolysis reaction, and the reaction of the functional cross-linker with hemoglobin gives only 40 % BT-Hb (71, 74). The rest was largely comprised of cross-linked hemoglobin that is not tethered through an inter-protein linkage. Thus, during the purification process used to obtain BT-Hb, the only hemoglobin contaminants (< 5 %) were cross-linked tetramers that do not dissociate.

Figure 4.2: Characterization of bis-tetramers (BT-Hb) and PEGylated bis-tetramers (BT-PEG). Size exclusion G-200 HPLC indicated that the high molecular weight BT-PEG was fully modified with high sample homogeneity.

In the preparation of BT-Hb and BT-PEG for animal studies, these materials must be oxygenated before administration to the animal. In order to minimize rapid auto-oxidation when the heme is exposed to oxygen, the Hb samples were stored frozen after oxygenation. However, upon thawing, it was observed that up to 20-25% of each sample were oxidized to the non-functional methemoglobin (metHb). This degradation of ferrous heme due to freeze-thawing has previously been observed, and this problem of auto-oxidation has been a problem in the design of HBOCs (83). The rate at which BT-Hb and BT-PEG auto-oxidizes to metHb is now currently under investigation. Nonetheless,

previous animal studies with high concentrations of metHb have shown that the presence of metHb does not significantly alter blood pressure levels, and HBOCs that elicit blood pressure increases continued to do the same even with up to 14.5 % metHb (82). We thus continued our blood pressure measurements with these compounds.

4.3.2 Hemodynamic Effects of Hemoglobin Solutions in Wild-Type Mice

Infusion of BT-Hb (4 g/dL) and BT-PEG (4 g/dL) did not cause systemic hypertension in awake WT mice (Figure 4.3A). The animals were not anesthetised, minimizing any possible external effects. Baseline control experiments with injections of modified PBS (n = 4) resulted in a constant systolic blood pressure (SBP) of 110 ± 3 mmHg. No change in blood pressure was observed over 1 hr. No increase in blood pressure from administration of either BT-Hb or BT-PEG was observed and the systemic blood pressure remained constant at 113 ± 5 mmHg after infusion of BT-Hb and similarly remained at 113 ± 4 mmHg after infusion of BT-PEG. In comparison, administration of murine tetrameric hemoglobin (mTet, 4 g/dL, n = 4) as a positive control resulted in an immediate increase in blood pressure (128 ± 3 mmHg) that was sustained over 1 hr after infusion ($P < 0.05$).

Figure 4.3: (A) Infusion of BT-Hb and BT-PEG through a tail vein injection into healthy WT mice did not cause significant differences in SBP (113 ± 5 mmHg and 113 ± 4 mmHg, respectively) compared to baseline injections of modified PBS (110 ± 3 mmHg), while injections of mTet resulted in a sustained increase in SBP (128 ± 3 mmHg). Even at a lower dosage (0.6xmTet), the increase in SBP (130 ± 5 mmHg) was observed. **(B)** Administration of BT-Hb and BT-PEG decreased HR transiently (447 ± 25 and 446 ± 28 beats/min respectively) but was recovered to baseline levels of modified PBS after 30 min. Injections of mTet resulted in a sustained decrease in HR (417 ± 17 beats/min) even after 1 hr of infusion.

Due to the high levels of methHb (~20 %) present in the hemoglobin samples used in the animal studies, we examined if the decreased vasoactivity of BT-Hb and BT-PEG could be the result of high methHb levels. Since ~20 % of the samples injected were non-functional methHb that cannot scavenge NO, the actual functional HBOC was effectively given at a lower dosage (~ 80 %). We thus injected a lower dose (0.18 mL instead of 0.3 mL in a 25 g mouse) of the positive control (mTet) and monitored blood pressure levels. Even at this lower dosage (60 % volume), 0.6xmTet (n = 6) elicited a strong increase in blood pressure (130 ± 5 mmHg) that was sustained over the course of the experiment (*P*

< 0.05, Figure 3a). This indicates that the lower effective dosage of BT-Hb and BT-PEG was not a contributing factor to their non-vasodilatory effect.

Effects on the heart rate were also studied (Figure 4.3B). The heart rate of healthy WT mice is typically 590 ± 10 beats/min. A transient decrease in HR was observed upon administration of BT-Hb ($n = 5$) and BT-PEG ($n = 5$) after 10 to 20 min infusion, where HR levels dropped to 447 ± 25 beats/min and 446 ± 28 beats/min, respectively. The HR normalized after 30 minutes and did not differ from baseline modified PBS injections. Infusion of the positive control, mTet ($n = 4$), elicited a sustained decrease in heart rate that was most apparent after 10 to 20 min of infusion (370 ± 25 beats/min), and remained low (417 ± 17 beats/min) even after 1 h.

4.3.3 Hemodynamic Effects of Hemoglobin Solutions in db/db Mice

Db/db mice with endothelial dysfunction have reduced endothelial NO bioavailability. They provide an excellent model to measure the level of Hb-mediated NO scavenging (80). We measured the change in SBP induced by intravenous infusion of BT-Hb and BT-PEG in db/db mice, and found that SBP did not differ from that following modified PBS injections (Figure 4.4A). In general, SBP increased from 119 ± 5 mmHg to 130 ± 9 mmHg transiently 10 to 30 minutes after infusion in all three samples. 30 minutes after infusion, SBP returned to 126 ± 5 mmHg for BT-Hb and 122 ± 9 mmHg for BT-PEG, and remained constant until the end of the experiment. In contrast, infusion of the same amount (0.48 g/kg) of murine tetrameric hemoglobin as a positive control shows a ~ 26 mmHg increase in blood pressure (80). It thus appears that even in mice that are extremely sensitive to changes in NO levels, both BT-Hb and BT-PEG do not disrupt important NO-signalling processes and do not alter steady-state blood pressure.

Figure 4.4: (A) SBP measurements in db/db mice with endothelial dysfunction. No significant change was observed between infusion of BT-Hb and BT-PEG compared to modified PBS control. The transient increase in SBP (10-30 min) was attributed to the large volume of fluids administered during experiment. (B) HR of db/db mice after infusion of samples. A small decrease of ~50 beats/min was observed for both BT-Hb and BT-PEG compared to baseline injections of PBS.

The transient increase of blood pressure is likely due to the stimulation of a venepuncture and large volume of fluids being IV infused into the animal as a “topload”. It thus appears that even in db/db mice that are extremely sensitive to NO scavenging by extracellular hemoglobin, both BT and BT-PEG did not disrupt important NO-signalling processes and did not alter systemic blood pressure. Although BT-Hb and BT-PEG did not affect SBP compared to the control infusion of modified PBS, a small decrease in HR was observed upon infusion of both these compounds (Figure 4.4B).

4.3.4 Plasma and Methemoglobin Levels

Two hours after infusion, mice were anesthetised through an intraperitoneal injection of Ketamine (120 mg/kg) and Xylazine (4 mg/kg), and oxygenated whole blood

was drawn through the left ventricle. Plasma hemoglobin levels and methemoglobin levels were analyzed (Figure 4.5).

Figure 4.5: Plasma hemoglobin levels and methemoglobin levels (WT in blue, db/db mice in mahogany). **(A, B)** After 2 hr infusion, 5% of mTet remained in the blood stream. Both BT-Hb and BT-PEG indicated a longer retention time in the circulatory system, 15% retained in WT mice and 20% retained in db/db mice. **(C,D)** Plasma methHb levels were increased upon infusion of BT-Hb (5%) and BT-PEG (9%) for both WT and db/db mice. Plasma methHb is calculated as a percentage of the total free plasma Hb.

Baseline infusion of modified PBS did not alter cell-free hemoglobin levels in the

plasma and only a small amount of cell-free hemoglobin was observed after infusion of PBS (1% in WT mice and 0.05% in db/db mice). In contrast, administration of BT-Hb, BT-PEG, and mTet increased the level of cell-free hemoglobin in the plasma. Both BT-Hb and BT-PEG remained in circulation ~ 3-4 times longer than did mTet. Modifications that stabilize the protein such as cross-linking and PEGylation thus decrease the rate of metabolism (through the liver), allowing the protein to circulate in the blood stream longer. This effect was observed in both WT and db/db mice.

Analysis of the plasma methemoglobin levels indicated that methHb levels were highest with BT-PEG, up to 10% in WT mice and db/db mice. Although the original solutions contained ~20-25 % methHb due to auto-oxidation, the low levels of plasma methHb (5 % in BT-Hb and 10 % in BT-PEG) after the infusion indicate that there is no significant further oxidation of the heme iron. It also appears that the healthy mouse was able to either remove or reduce the ferric methemoglobin within the course of the experiment. This may come from the addition of the reducing agent – *N*-acetyl cysteine as an additive in the PBS used to dissolve the hemoglobin.

4.4 Discussion

4.4.1 Bis-Tetramers and PEGylated Bis-Tetramers: Lack of Vasoactivity

We have now examined the systemic hypertensive effects of both BT-Hb and BT-PEG in awake and anesthetized wild-type C57BL/6J mice and found that no systemic vasoconstriction occurs with these compounds compared to baseline injections of modified PBS. Similarly, experiments with obese mice (db/db mice), which have reduced endothelial NO bioavailability, demonstrated that infusion of BT-Hb and BT-PEG do not alter SBP significantly. Db/db mice exhibit an enhanced vasoconstrictor response and thus serve as a sensitive model for HBOC-induced toxicity (80). We observe no HBOC-induced hypertension in both healthy WT and db/db mice.

Differences in HR after infusion of HBOCs indicate that there may be stress induced by BT-Hb and BT-PEG on the blood vessels. A transient decrease in HR is observed in healthy WT mice while a sustained decrease in HR (~50 beats/min) is

observed in db/db mice. The lowered HR likely comes from the baroreflex that alleviates stress on the endothelial wall. It appears that healthy mice are able to recover from the strain on blood vessels within the course of the experiment, while db/db mice with endothelial dysfunction do not. It is unclear at this point, if the observed bradycardia will not be detrimental to the animal, since it may reflect cardiac toxicity.

4.4.2 Sample Purity and Homogeneity

The lack of hypertension observed from both of the potential HBOCs appears to give support to the validity of HBOCs based on structurally defined high molecular weight materials with appropriate oxygen-binding characteristics as described earlier. Current methods for increasing molecular weight are summarized in Table 4.2 (12, 38, 61, 71, 74).

Table 4.2: Physical Properties of High Molecular Weight HBOCs

	HBOC-201 (38)	PolyHeme (84)	MP4 (50)	BT (74)	BT-PEG (74)
Modification	Polymerized	Polymerized Pyridoxylated	PEGylated	Cross-linked	Cross-linked PEGylated
MW (kDa)	250	150	94	128	148
% Tet	≤ 3	≤ 1	100	0	0
P_{50} (mmHg)	32-38	26-32	3.7	9.3	4.1
n_{50}	~ 1	~ 1	1.1-1.8	2.7	2.4
SBP (WT)	Increased	No change	No change	No change	No change

Although polymerization and PEGylation methods result in high molecular weight materials (250-94 kDa), these are heterogeneous mixtures (38). HBOCs of a heterogeneous nature not only result in a large range of oxygen affinity (Hemopure, P_{50} = 32 - 38 mmHg (85)) but also are completely lacking cooperativity ($n_{50} \sim 1$). They are thus ineffective at delivering oxygen to the tissues in an accessible range and have the potential of toxicity from tetramers contained in the mixtures. These undefined can still

interact with the endothelium and interstitia. In contrast, BT-Hb and BT-PEG are prepared in a way that eliminates by-products, making them free of smaller species.

4.4.3 Favourable Oxygen Binding Properties

Since HBOCs are designed to serve as alternative to red cells in transfusions, they should transport oxygen in a manner that effectively works with RBCs. Among higher molecular weight HBOCs, only BT-Hb ($n_{50} = 2.7$) and BT-PEG ($n_{50} = 2.4$) retain good cooperativity (74), which is necessary for effective oxygen circulation. This comes from cross-linking the tetrameric protein at sites that do not interfere with R to T state conformational changes (86).

In our design for functional bis-tetramers, we have found that the structure of the inter-protein linkage between cross-linked hemoglobin tetramers affects cooperativity (71, 73). Linkages with torsional flexibility from a tetrahedral centre, such as is found in a sulfone or ether linkage, give materials with much higher Hill coefficients ($n_{50} = 2.5$ -2.7) compared to their linear analogues ($n_{50} = 1.5$ -1.8). The higher cooperativity results in enhanced potential for oxygen delivery (71). In contrast, heterogeneously polymerized HBOCs have oxygen binding curves that are hyperbolic, indicative of a non-cooperative ligand binding process (9).

The HBOC's affinity for oxygen is also crucial for correct delivery of oxygen. BT-Hb and BT-PEG have relatively high oxygen affinities ($P_{50} = 9.3$ and 4.1, respectively). Both these materials are thus expected to only give up oxygen at low oxygen partial pressures, complementing the delivery of oxygen from RBCs. This comes from the disparate modes of oxygen transport by large RBCs versus small, diffusible oxygen carriers. The release of oxygen from RBCs is driven by the difference of oxygen partial pressure between the high P_{O_2} of blood and the low P_{O_2} of tissues. The decrease hematocrit near the vessel wall results in a no-RBC layer, forming a diffusional barrier and increased distance for oxygen diffusion to tissues (98). In contrast, a small highly diffusible cell-free hemoglobin can increase lateral transport by acting as stepping stones for oxygen delivery from RBCs (101). This is the basis of HBOC facilitated diffusion. It has been mathematically shown that the facilitated flux of oxygen depends on the initial

concentration of the carrier protein, its diffusion coefficient (molecular size) and its rate of oxygen dissociation (oxygen affinity) (101). This phenomenon has now been demonstrated in vivo in a hamster skinfold model (23) and through simulations with arteriolar-size conduits (98). The high oxygen affinity and increase molecular size of both BT and BT-PEG may have the necessary properties that reduce pre-capillary oxygen off-loading in a similar manner that has been demonstrated by **MP4OX**. However, this has yet to be experimentally proven.

4.4.4 Nitrite Reductase Activity at Physiologically Relevant Sites

We have also previously examined the nitrite reductase activity of BT-Hb and BT-PEG in comparison to other existing HBOCs and found that the correlation between nitrite reductase activity and the oxygen binding properties (P_{50} and n_{50}) may play a significant role in making these materials non-vasoactive (60). The site at which the rate of NO production is fastest depends directly on the HBOC's P_{50} (64, 90). BT-Hb and BT-PEG both have high oxygen affinities (P_{50} = 9.3 and 4.1 respectively). Thus, maximal NO production will occur at tissue sites with low pO_2 , counteracting NO scavenging by HBOCs. As well, both these compounds have increased rates of nitrite reduction, producing NO at faster rates than native hemoglobin. A combination of enhanced nitrite reduction at physiologically relevant sites may be a contributing factor to making these materials non-hypertensive.

4.5 Summary

We have produced bis-tetramers and PEGylated bis-tetramers of hemoglobin and have shown that they do not cause increases in blood pressure in murine models. The lack of induced hypertension is consistent with their size, their cylindrical structures, their appropriate oxygenation properties and their enhanced nitrite reductase activity. These characteristics suggest that production and utilization of safe and effective HBOCs for clinical evaluation can be achieved.

Chapter 5 : Conclusions and Further Work

5.1 Conclusions

HBOCs have been developed as alternatives to blood transfusions for use as oxygen-bridging agents (17, 24). However, the complications observed in clinical trials of HBOCs have raised questions about their safety (12, 41). Evaluations of the characteristic and clinical profiles of these products indicate several prominent features that are important for a functional HBOC. Processes that affect NO homeostasis are particularly important for proper vascular blood tone. Hemoglobin was originally believed to interact with NO in a manner that would inactivate its oxygen-carrying capacity (13, 91). However, hemoglobin-mediated nitrite reduction and vasodilation has now been supported by *in vitro* biochemical (60, 74) and aortic ring bioassay studies and by *in vivo* nitrite infusions in human volunteers (16). Of course, any potential benefit from enhancement of the nitrite reductase activity of altered hemoglobin requires that the resulting NO avoid reaction with residual free deoxy-hemes within the same protein (57).

As previously reviewed, a study of the reaction mechanism reveals the presence of metastable intermediates that provide a regulated source of bioactive NO (68, 92). In addition, the nitrite reductase-anhydrase redox cycle has been shown to yield di-nitrogen trioxide (N_2O_3), a stable intermediate that provides a pathway that avoids reaction between NO and ferrous heme. N_2O_3 can then revert to NO and NO_2^- , acting as a circulating source of bioactive NO (93). As well, the simultaneous interaction between the oxidative oxyHb-nitrite reactions and the deoxyHb-nitrite reduction results in oxidative de-nitrosylation that provides an escape route for NO after its formation (55).

In this work we demonstrate the effects of various methods of hemoglobin cross-linking and conjugation on the protein's nitrite reductase activity (60). We show that PEG-hemoglobins react at enhanced rates to produce NO, a reaction that is related to the protein's affinity and cooperativity for oxygen binding and release. We have also further produced BT and BT-PEG as purified compounds that are chemically well-defined (74).

The homogenous nature of these materials is particularly important for the assessment of reproducible physiological responses in pre-clinical animal studies. They have high oxygen affinity and excellent cooperativity combined with an enhanced nitrite reductase activity. Their large size will minimize the transcapillary movement out of the blood vessel, where a smaller HBOC would scavenge NO within the interstitial spaces between smooth muscle cells (16).

We also find a correlation between nitrite reductase activity and the protein's oxygen binding properties (P_{50} and n_{50}) and this may play a significant role in making these materials non-vasoactive (60). The circulatory site at which the protein's nitrite reductase activity is maximal correlates with the deoxygenation of the protein (P_{50}) (64, 90). BT-Hb and BT-PEG are expected to produce NO at physiologically relevant sites with low pO_2 .

We further demonstrated that infusions of both BT and BT-PEG do not cause significant increases in systemic blood pressure in mice. This effect is observed in both healthy wild-type (WT) mice as well as in obese db/db mice that are highly sensitive to NO scavenging. The lack of hypertension is likely due to the absence of tetramers, their cylindrical structures, their appropriate oxygenation properties, and their enhanced nitrite reductase activity. These characteristics suggest that production and utilization of safe and effective HBOCs for clinical evaluation can be achieved.

5.2 Further Work: Hemoglobin bis-tetramers *via* cooperative azide-alkyne coupling

BT and BT-PEG are prepared in a chemical process that eliminates by-products, making them completely homogenous. The high purity of these compounds comes from the specific acylation reactions within the BPG binding site of the protein (71). Nonetheless, due to a competing ester hydrolysis reaction, bis-tetramers are only formed in 40% yield. The reaction mixture must then be extensively purified in order to isolate pure bis-tetramers. The yield of this reaction is a significant drawback and will create difficulties for the scale-up production of this product.

In order to avoid competitive hydrolysis, we have now designed reagents that make use of copper-catalyzed azide-alkyne coupling reactions (“click chemistry”) (94).

Instead of introducing the azide functionality through the complex method of site-directed mutagenesis, we introduced the azide group through direct addition of an azide-activated cross-linker (**Appendix 5.1**). Bis-tetramers of hemoglobin can be produced by coupling a single azide-functionalized, cross-linked hemoglobin tetramer that would then react with a bis-alkyne to link the proteins (Figure 5.1).

Figure 5.1: Reaction of the hemoglobin tetramer with azide activated cross-linker (step 1) yields a hemoglobin azide (Hb- N_3 , JF-1). Coupling of the hemoglobin azide with a bis-alkyne (step 2) produces a bis-tetramer of hemoglobin. This coupling reaction is catalyzed by copper.

Thus, we synthesized and characterized a modified Hb containing a cross-linker with an azide (Hb- N_3) and developed a route to coupling two of these to a single bis-alkyne (94). Although the copper-catalyzed azide-alkyne coupling reaction (Step 2, Azide-Alkyne Coupling) is highly efficient (95), bis-tetramers are still not formed in high

yields (20-25 %) (94). Analysis of the reaction products of the combination of the cross-linking material with hemoglobin showed that the reagent is not selective as it reacts at lysyl amino groups in both the α and β subunits (Step 1, Hemoglobin Cross-linking). The diester reacts with deoxyhemoglobin to give two major hemoglobin containing products, one with an azide-containing cross-link between the α -subunits and the other with the cross-link between the β -subunits. While hemoglobin with the azide-containing cross-link within the β -subunits undergoes reaction with a dialkyne, the $\alpha\alpha$ -cross-linked derivative does not react with the dialkyne, presumably because the azide is inaccessible for the reaction (95).

A method that deals with this problem comes from designing an azide-activated cross-linker that is specific for the $\beta\beta'$ -BPG binding site. If full modification occurs within the $\beta\beta$ -subunit, this Hb-N₃ will be accessible for further reaction, possibly allowing the overall coupling reaction to have quantitative yields. A promising approach comes from using a known $\beta\beta'$ -directing cross-linker that has previously been produced in our lab (96). Trimesoyl tris(3,5-dibromosalicylate) (TTDS) reacts selectively with the ϵ -amino groups of Lys-82 of each of the β -subunits of hemoglobin (96). It is likely that the selectivity arises from the three anionic ester groups directing reaction to the cationic region at the surface of the BPG-binding site between the β -subunits. An azide cross-linker based on the structural and electronic features of TTDS should potentially result in the same kind of selectivity observed with TTDS.

Molecular modelling of TTDS and comparison with the first generation HbN₃ cross-linker (JF-1) revealed that they have very different low energy conformations (Figure 5.2). The symmetry of the DBS groups in TTDS and the repelling force of the three anionic ester groups constrain the molecule within a relatively co-planar position, with all three ester groups equi-distance from each other. In contrast, the reversal of the carbonyl group in JF-1 introduces a certain amount of flexibility into the molecule, allowing for a π - π stabilizing effect to take place. This position of the amide bond was originally chosen because it is more readily synthetically accessible.

Figure 5.2: (A, C) Chemical and molecular modelling structure (as calculated in Spartan, Molecular Mechanics) of TTDS indicate a rigid conformation with all three ester groups equi-distance from each other. (B,D) Chemical and molecular modelling structure of JF-1 depicting the smaller size of the more stable *cis*-amide conformation.

It is possible to envision that reversing the position of the carbonyl group will decrease flexibility, forcing the cross-linker into a more rigid position. Molecular modelling indicates that the reversal of the carbonyl group will not only prevent π - π stacking, it will give an amide bond that is in a position for favourable hydrogen-bonding, thus making the *trans*-amide the more stable conformer (Figure 5.3).

Figure 5.3: Chemical structure and molecular modelling of a cross-linker with reversal of the amide bond is in a position to hydrogen bond to the carboxylate groups, stabilizing the *trans*-amide conformer, and forcing the overall molecule into a rigid conformation.

If this cross-linker yields a site-specific $\beta\beta'$ -cross-linking reaction, it can be envisioned that quantitative yields of bis-tetramers via azide-alkyne click chemistry can be achieved. This is extremely important in the goal towards commercializing these materials that have already been shown to have certain features conducive towards a safe, functional HBOC.

5.3 Outlook

It still remains a challenge to find a favorable balance between the risks and benefits for hemoglobin-based oxygen therapeutics and to design appropriate methods for evaluating the efficacy and safety of HBOCs in clinical situations. A way forward for a red-cell substitute will require thorough understanding of how cell-free hemoglobin affects oxygen and NO homeostasis responses. The importance of counteracting NO scavenging might be of central importance, yet it is a complex process that needs to be understood. The relationship between the redox potential of altered hemoglobins and their heme-nitrite rates will further provide information about the factors that govern the protein's nitrite reductase activity. Bis-tetramers of hemoglobin and their PEGylated derivatives have various favorable features. Optimization of the azide-alkyne coupling method will encourage further study of these materials. For the years to come,

understanding processes that contribute to HBOC-induced vasoconstriction and designing strategies to overcome toxicity issues will be a necessary approach for the development of safe and effective transfusion alternatives.

Appendix

2.1 HPLC Analysis – Buffer Gradient

Solvent A: 0.1% TFA, 20% Acetonitrile in doubly distilled water

Solvent B: 0.1% TFA, 60% Acetonitrile in doubly distilled water

Elution Gradient for Analytical Column

Time (min)	v/v% A	v/v% B
0.00	49.0	51.0
0.50	49.0	51.0
60.50	35.0	65.0
80.50	14.0	86.0
95.00	0.0	100.0
105.00	0.0	100.0
106.00	49.0	51.0
120.00	49.0	51.0

2.2 CD Spectroscopy

Globin chain secondary structure change

Wavelength: 203-260 nm

Heme conc: 5 μ M

Buffer solution: sodium phosphate buffer $I = 0.02$ M, pH 7.4

Solution volume: 10 mL (make up in 10 mL vol flasks)

Cell: L=2 cm

Parameter:

Sensitivity: 20 mdeg

Resolution: 1 nm

Bandwidth: 1.0 nm

Response: 2sec

Scan speed: 200 nm/min

Accumulation: 1 (or 3)

Heme microenvironment

Wavelength: 245-470 nm

Heme conc: 50 μ M

Buffer solution: sodium phosphate buffer $I = 0.02$ M, pH 7.4

Solution volume: 10 mL (make up in 10 mL vol flasks)

Cell: L=2 cm

Parameter:

Scan scale: 245-470 nm

Sensitivity: 10 mdeg

Step Resolution: 2 nm

Bandwidth: 2.0 nm

Response: 2 sec

Scan speed: 200 nm/min

Accumulation: 1 (or 3)

2.3 Thiol Determination with 5,5'-dithiobis(2-nitrobenzoate) (DTNB)

DTNB Assay with 1 mL reaction volume

1. Dissolve 7-8 mg DTNB in 700 μL buffer (bis-tris buffer, 0.05 M, pH 7.2), and 300 μL 1 M potassium phosphate (pH 8.0). Make mix with DTNB and another mix WITHOUT DTNB (to do this, replace DTNB volume with buffer). The mix without DTNB is made because hemoglobin absorbs at 412 nm as well, and the baseline hemoglobin spectrum needs to be subtracted out (refer to FL-Labbook 1, DTNB).

Mix with DTNB

Mix (μL)	1 reaction	10 reactions	15 reactions	20 reactions
Buffer	750	7500	11,250	15,000
0.5 M EDTA	2	20	30	40
DTNB	15	150	225	300
Total	767	7670	11,505	15340

2. First dilute 10 μL of BME in 990 μL buffer. Vortex to mix. Dilute 10 μL of first dilution of BME in 990 μL buffer, and vortex.

Standard

	Mix (μL)	Buffer (μL)	Diluted BME
0 (blank)	765	235	0
1	765	230	5 (7.15 μM)
2	765	225	10 (14.3 μM)
3	765	215	20 (28.6 μM)
4	765	205	30 (42.9 μM)
5	765	195	40 (57.2 μM)

Sample

	Mix (μL)	Buffer (μL)	Sample
1	765	220	15
2	765	215	20
3	765	210	25
4	765	205	30

3. Measure at 412 nm. Make sure that samples will fall into range of standards! Check concentrations of samples. Pipette up and down to mix. Hb conc at final should be 6 μM , or above, for this conc, do studies between 10-20 μL .

4. Also make samples with mix WITHOUD DTNB so that you can subtract out the Hb absorbance.

2.4 Hemoglobin-Nitrite Kinetic Measurements

Procedure for Hb + NO₂⁻ Reaction

Concentration of Hb in Bis-Tris Buffer (0.05 M, pH 7.2):

Need x μ L Hb + y μ L Bis-Tris to make 0.03-0.05 mM Hb (Hemoglobin solution must be more concentrated than final concentration. Hemoglobin concentration is determined using the cyanomethemoglobin method. This method of dilution is the most accurate way of obtaining the correct concentration of hemoglobin)

1. x μ L Hb (in Bis-Tris) is added to y μ L Bis-Tris (to make 1.5 mL total volume) into a 2-neck 10 mL round bottom flask and left to oxygenate for 2 hr.
2. During this time, make up a 10 mM sodium nitrite solution in bis-tris buffer.
3. Hb mixture is switched to deoxygenate for 2 hr. Degass 10 mM nitrite solution, and a round-bottom-flask filled and stoppered with ddH₂O with nitrogen (this is for syringe purging) for 2 hr. This makes the solution oxygen-free. Sealed cuvette is also flushed with nitrogen and left to sit for 2 hr.
4. Before hemoglobin deoxygenation is finished, set up computer in advance. (Follow procedure on FL-Labbook 2 pg 35, **Remember to record the start time**). Meanwhile, Open Spectra 1.7, under Application, go to time studies. Set wavelength scan to be 500-650 nm, for 3600s in 30s scans. After spectrometer has warmed up (15 min), run baseline.
5. After the 2 hr deoxygenation of hemoglobin, PURGE gas-tight syringes by taking up nitrogen from the headspace in water filled RBF (the water helps with the bubbling of nitrogen). Once syringes are purged with nitrogen and oxygen-free, take up 1 mL of deoxyHb mixture and add into sealed cuvette. Take 25-100 μ L sodium nitrite, add into cuvette and insert into UV-vis.
6. Date, and record all changes/observations to procedure in labbook.

Procedure for washing up

1. For syringes, rinse with distilled water. Take the needle and plunger apart. Wash all the parts with water. Wash the needle and body with acetone (NEVER THE PLUNGER). Flush dry with air.
2. For cuvette, rinse with distilled water. (NEVER with acetone). Leave soaking in water overnight then flush dry with air.
3. For 2-neck flask, rinse with water and acetone and then leave to open air to dry.

2.5 Deconvolution of Spectra and Processing of Data

Processing of Data

To process data saved from UV-Vis spectrometer (data saved as .ASC file), use Excel's macros to cut and paste data (e.g.):

```
ActiveCell.Range("A1:A284").Select
Selection.Cut
ActiveCell.Offset(0, 2).Range("A1").Select
ActiveSheet.Paste
ActiveCell.Offset(0, -2).Range("A1:A284").Select
Selection.Delete Shift:=xlUp
Selection.Cut
ActiveCell.Offset(0, 3).Range("A1").Select
ActiveSheet.Paste
ActiveCell.Offset(0, -3).Range("A1:A284").Select
Selection.Delete Shift:=xlUp
```

This gives the time changes in absorbance over the wavelength range 500 nm – 650 nm. A time scan graph such as in Figure 2.5A and 3.6A can thus be generated.

Deconvolution of Data

To deconvolute the spectra data and generate a reaction profile graph such as in Figure 2.5C-E and Figure 3.6C-F, save all data numbers (without wavelength numbers, only Abs numbers) generated from excel's macros into a notepad file (e.g. **Jan29-50.txt**) in a specific folder (e.g. data for R).

Open R.cran and go to change directory. Here, open the folder that the program LS_deoxyNO2.R is written (e.g. Data for R).

#Open R and type:

```
#source("LS_deoxyNO2.R")
```

The code for LS_deoxyNO2.R is as follows (This code requires the files k-values.txt, which is the molar absorbtivity of oxy, deoxy, met and NOHb. It also requires the numbers from spectral scans, e.g. **Jan29-50.txt**):

```
k_all = as.matrix(read.table("k-values.txt", sep="\t", header=FALSE))
abs_all = as.matrix(read.table("Jan29-50.txt", sep="\t", header=FALSE))

randm = sample(1:nrow(k_all), 200)
k_all = k_all[randm, ]
abs_all = abs_all[randm, ]

fit = matrix(data = NA, nrow = ncol(abs_all), ncol = ncol(k_all))
res = matrix(data=NA, nrow=nrow(abs_all), ncol=ncol(abs_all))
for(i in 1:ncol(abs_all)) {
  #select abs at time i
  abs = abs_all[,i]
  ls = lsfit(k_all, abs, intercept=FALSE);
  fit[i, ] = as.vector(ls$coef)
```

```

        res[, i] = as.vector(ls$residuals)
    }
    min = matrix(apply(fit, 2, min), nrow=nrow(fit), ncol=ncol(fit), byrow=TRUE)

    fit = fit - min

    plot(fit[, 1], col="red", type="l")
    lines(fit[, 2], col="blue")
    lines(fit[, 3], col="green")
    lines(fit[, 4], col="orange")

    write.table(fit, "decon.txt", row.names=FALSE, col.names=FALSE,
    quote=FALSE, sep="\t")

```

After R.cran has generated the plots, the new deconvoluted data is saved as decon.txt in the original folder (e.g data for R) in a notepad file. These numbers can be transferred to Excel or GraFit and the reaction profile plotted.

5.1 Hemoglobin bis-tetramers *via* cooperative azide-alkyne coupling

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Note: The concepts and experimental design were conducted by Jonathan S. Foot and Ronald Kluger. Most experimental work was carried out by Jonathan S. Foot with contributions from Francine E. Lui. Data interpretations and writing of the manuscript was carried out by Jonathan S. Foot and Ronald Kluger with contributions from Francine E. Lui.

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